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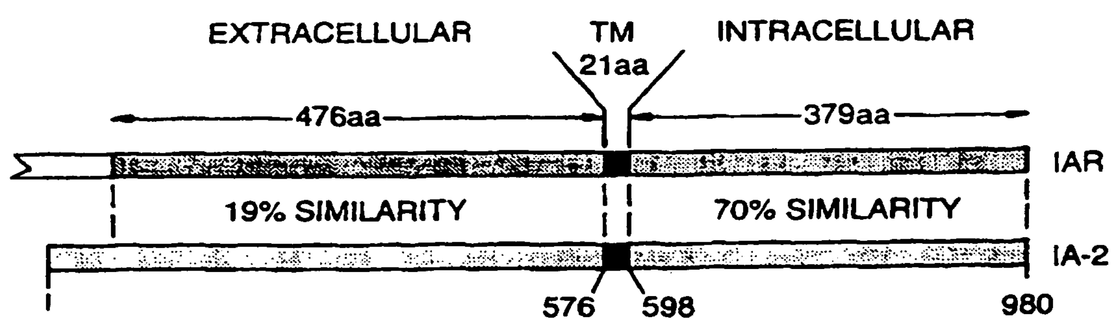


(57) Abstract

The present invention relates to a nucleic acid encoding a polypeptide that has the properties of an Insulin-dependent diabetes mellitus (IDDM)-associated autoantigen, which nucleic acid comprises: (a) the coding sequence of SEQ. ID. No. 1, 10 or 11, and/or the sequence complementary thereto; (b) a sequence which hybridises to a sequence defined in (a); (c) a sequence that is degenerate as a result of the genetic code to a nucleic acid sequence defined in (a) or (b); (d) a sequence having at least 80 % homology to a sequence defined in (a), (b) or (c). It also relates to the polypeptides encoded by such nucleic acids, and to diagnostic methods which employ such polypeptides.

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# DIAGNOSTIC REAGENTS RELATING TO DIABETES

The present invention relates to an autoantigen associated with type I diabetes (Insulin Dependent Diabetes Mellitus (IDDM)). This autoantigen is useful in the diagnosis of IDDM.

IDDM is an autoimmune disease. It is caused by the autoimmune system-mediated destruction of pancreatic islet beta-cells. This leads to an imbalance in the levels of the hormones insulin and glucagon which disrupts glucose metabolism, leading to the well-known symptoms of diabetes, such as high glucose levels in urine. As IDDM is an autoimmune disease, autoantigens can often be detected in the blood of individuals suffering from IDDM.

Some characterisation of the autoantigens involved in IDDM has already been achieved. In particular, two autoantigens are known to be closely associated with the development of IDDM; these are known as the 37 kDa and 40kDa autoantigens, because of their respective molecular weights. It has recently been suggested that the 40kDa autoantigen is a protein known as IA-2 (Payton *et al* (1995) J. Clin. Invest. 96, 1506-1511).

Human IA-2 (hIA-2) was originally cloned in a truncated form, and given the name ICA-512 (Islet Cell Antigen 512) (Rabin *et al* (1994) J. Immunol. 152, 3183). Subsequently, the complete DNA and amino acid sequences were determined and the protein was given the name IA-2 (Lan *et al* (1994) DNA and Cell Biology vol 13, No 5, pp 505-514).

A mouse homologue (mIA-2) is also known (Lu *et al* (1994) Biophys. Biochem. Res. Commun. vol 204, No 2). Its cDNA shows 92% identity to that of hIA-2. Two possible rat homologues have also been identified. The first of these is known as PTPLP (Kambayashi *et al* (1995) Biochem. J. 306, 331-335) and has 79.3% identity to hIA-2 at the amino acid level. The second has a sequence identified from a series of overlapping clones (Passini *et al* (1995), Proc. Natl. Acad. Sci. USA 92, 9412-9416). This DNA shows 93% homology

to the corresponding mIA-2 sequence and 86% homology to the corresponding hIA-2 sequence.

As stated above, it has been suggested that hIA-2 is the 40kDA autoantigen associated with IDDM.

Other putative IDDM-associated autoantigens include glutamic acid decarboxylase (GAD) and the islet cell autoantigens (ICA) known as ICA-12, ICA-13, ICA-208, ICA-302, ICA-313, ICA-123, ICA-525 and ICA-505 (EP-A-0, 383, 129; EP-A-0, 569,800).

Some of these IDDM-associated antoantigens have been proposed as diagnostic reagents for screening patients for IDDM, notably GAD, ICA-512 and ICA-12 (EP-A-0 569, 800).

Whilst searching for protein tyrosine phosphatases, the present inventors unexpectedly cloned and sequenced a cDNA fragment, and then a complete cDNA, encoding part of a new probable IDDM-associated autoantigen. This has been give the name IAR (Islet Cell Antigen-Related) and may also be referred to as IAR-PTP (Islet Cell Related Protein Tyrosine Phosphatase).

Analysis of this partial sequence shows that, like the known autoantigens mentioned above, it has an extracellular region and an intracellular region. The partial IAR sequence encodes around 460 amino acids from the extracellular domain and the whole of the intracellular domain. It is likely that the intracellular domain is an antigenic part of the protein.

The IAR sequence identified by the inventors shows around 70% nucleotide and amino acid sequence homology to the IA-2 sequence in the intracellular region. This provides further evidence that IAR is an IDDM-associated autoantigen. In the extracellular region, the nucleotide homology to IA-2 is about 35% and the amino acid sequence homology is about 20%.

Although previous work has suggested that hIA-2, PTLTP and mIA-2 do not have protein tyrosine phosphatase (PTP) activity, the present inventors have found that hIA-2 in fact has low but detectable PTP activity. Like hIA-2, IAR

has PTP activity, and is also referred to herein as IAR-PTP (Islet Cell Antigen-Related Protein Tyrosine Phosphatase).

Further, IAR has a similar tissue expression pattern to IA-2, being highly expressed in the brain and pancreas. Thus, IAR has three characteristics of an IDDM-associated autoantigen: (i) a characteristic structure, in terms of organisation into intra- and extra- cellular domains and the sequence of its intracellular domain; (ii) PTP activity; and (iii) a characteristic tissue distribution.

IAR is also reactive with IDDM patient sera. A study with a group of IDDM patients shows that recombinant IAR is precipitated by autoantibodies present in more than 50% of the serum samples while IAR does not react with any sera from control patients.

Nonetheless, it is clear that IAR is distinct from previously isolated autoantigens; in particular, its sequence identity to hIA-2, mIA-2 and PTPLP is too low for it to be any of these proteins, especially as it has only 20% homology to hIA-2 at the amino acid level in the extracellular region. Further, IAR and IA-2 can distinguish different populations of IDDM autoantibodies.

The inventors have also carried out a study to compare the IDDM sensitivity and predictive value of IAR antibodies (IAR Ab) and IA-2 antibodies (IA-2 Ab). Antibodies to IAR and IA-2, measured by radiobinding assay, were correlated in recent-onset IDDM patients. Some individuals had IAR Ab in the absence of IA-2 Ab, and others had IA-2 Ab in the absence of IAR Ab. Therefore, IAR and IA-2 can distinguish different populations of IDDM autoantibodies since they identify overlapping but non-identical sets of IDDM patients. The diagnostic sensitivity for IDDM was similar for IAR Ab and IA-2 Ab. Accordingly, IAR is clearly a useful predictive tool for the diagnosis of IDDM, either as an alternative IA-2 or in combination with IA-2.

Moreover, in a group of first degree relatives of IDDM sufferers, the presence of IAR Ab were significantly associated with progression to IDDM while IA-2 Ab were not.

This is significant because it establishes that IAR Ab are likely to be better predictors of IDDM than IA-2 Ab in first degree relatives of IDDM sufferers, who are a high risk group for contracting IDDM. Therefore, the IAR of the invention is likely to be effective in predicting the onset of IDDM in high-risk groups such as first-degree relatives.

Further, the inventors have found that the cytoplasmic (intracellular) domain of IAR is itself a useful predictor of IDDM. It is likely to be as useful as, or more useful than, complete IAR.

A brain Expressed Sequence Tag (EST) is available which has a sequence corresponding to part of the IAR sequence and highly homologous to the human IA-2 sequence. This is EST03250 (Adams *et al*; Nature Genetics Vol. 4, July 1993). The length of the EST is 313 nucleotides. It was identified during the partial sequencing of over 3400 ESTs from human brain cDNA, in an attempt to demonstrate the diversity of RNA transcripts present in brain cells. IAR of course differs in terms of sequence from this EST because it has a different, longer, sequence. Also, it is unknown whether or not the EST encodes a functional autoantigen.

IAR sequences of the invention can be used in the diagnosis of IDDM, for example to screen for or predict the onset, presence or development of IDDM. For example, individuals who have, or are predisposed to develop, IDDM can be expected to have autoantibodies to the autoantigenic IAR in their blood. Thus, blood, serum or other samples can be taken and contacted with the IAR of the invention. The anti-IAR autoantibodies will recognise the exogenous IAR and this recognition can be detected, for example by detecting the IAR/anti-IAR complex thus formed.

Accordingly, the present invention provides:

A nucleic acid encoding a polypeptide that has the properties of an Insulin-dependent diabetes mellitus (IDDM)-associated autoantigen, which nucleic acid comprises:

(a) the coding sequence of SEQ. ID. No. 1, 10 or 12 and/or the sequence complementary thereto;

(b) a sequence which hybridises to a sequence defined in (a);

(c) a sequence that is degenerate as a result of the genetic code to a nucleic acid sequence defined in (a) or (b);

(d) a sequence having at least 80% homology to a sequence defined in (a), (b) or (c);

a polypeptide encoded by such a nucleic acid;

a nucleic acid vector including such a nucleic acid;;

a cell harbouring such a vector;

a method of diagnosing IDDM which comprises:

(a) contacting a sample from an individual with a polypeptide as defined above under conditions that permit the recognition of the polypeptide by autoantibodies that recognise IAR; and

(b) determining whether or not said recognition occurs.

a method of producing a polypeptide as defined above which comprises culturing a cell as defined above under conditions which permit the expression of the polypeptide; and recovering the polypeptide;

use of a polypeptide as defined above in diagnosing IDDM;

a diagnostic test kit for diagnosing IDDM which comprises a polypeptide as defined above, and means for determining whether or not the polypeptide is recognised by autoantibodies that recognise IAR.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1. Northern analysis of r75 tissue expression.** Human multiple tissue Northern blots are from Clontech and the source of the mRNA in each lane is indicated at the bottom of the figure. The r75 probe was labelled with $^{32}$P-dCTP (Amersham) using the High Prime DNA Labeling Kit (Boehringer Mannheim). Hybridizations were carried out at

65°C overnight in 0.5M sodium phosphate (pH 6.8), 7% SDS, 15% formamide, 1mM EDTA. After washing 3 times with 50mM sodium phosphate (pH 6.8), 1% SDS for 40 min at 65°C, the blots were exposed to film at -70°C. Blots were stripped of probe by incubation in freshly boiled 0.1% SDS (3 x 2 min) before reprobing. Top panels, r75; bottom panels, β-actin. The positions of molecular size markers (in kb) are shown to the left of each blot.

**Figure 2. Schematic depiction of IAR clones and predicted protein product.**

A. Overlapping cDNA clones C3 and 3-7. Nucleotide numbering is as in SEQ. ID. No.1.

B. Predicted IAR protein product aligned with the IA-2 protein. The predicted partial IAR polypeptide (top) translated from and aligned with the clones shown in A is depicted in alignment with the IA-2 protein (Lan *et al.*, 1994). The % amino acid similarity between the intracellular regions of IAR and IA-2 is shown, as is that between the partial extracellular region of IAR and the corresponding portion of IA-2. TM, transmembrane region.

C. Relationships of cDNA clones. The relative positions of clones B11, C3, 3-7 and 3-10 are shown. The coding portion of these clones is represented by the thick line on the top scale. Both strands of clones B11, C3, and 3-7 were sequenced in their entirety. The angled 5′ piece of C3 is not identical to the corresponding region of B11 and likely represents a cDNA library artifact, as does the boxed region (54 bp) of C3 which is a sequence found in the opposite orientation in the corresponding region of B11.

**Figure 3. Northern analysis of IA-2 tissue expression.** Human multiple tissue Northern blots (different membranes from those used for Fig.1) are from Clontech and the source of the mRNA in each lane is indicated at the bottom of the figure. The IA-2 probe (nucleotides 1590-1906, coding for a portion of the extracellular region, the transmembrane

region, and a small portion of the intracellular region; and non-homologous to IAR sequence) was labelled and hybridization carried out as described in the legend to Fig. 1. The position of the IA-2 transcript is indicated by the arrow at the right of the figure. The $\beta$-actin signal (position indicated by the parentheses to the right of each panel) results from a previous hybrization with a $\beta$-actin probe, and could not be completely stripped from the membranes before reprobing with IA-2. The positions of molecular size markers (in kb) are shown to the left of each blot.

**Figure 4. Northern analysis of IAR tissue expression.** Human multiple tissue Northern blots are from Clontech and the source of the mRNA in each lane is indicated at the bottom of the figure. Membranes were stripped of IA-2 probe (Fig.2) by incubation in freshly boiled 0.1% SDS (3 x 2 min). The IAR probe (nucleotides 790-1197 of SEQ. ID. No.1, coding for a portion of the predicted extracellular region and non-homologous to IA-2 sequence) was labelled and hybridization carried out as described in the legend to Fig. 1. The positions of IAR transcripts are indicated by the arrows at the right of the figure. The $\beta$-actin signal (position indicated by the parentheses to the right of each panel) results from a previous hybrization with a $\beta$-actin probe, and could not be completely stripped from the membranes before reprobing with IAR. The positions of molecular size markers (in kb) are shown to the left of each blot.

**Figure 5. Comparison of IAR and IA-2 transcripts in human brain and pancreas.** The indicated lanes from the Northern analyses of IA-2 (Fig. 3) and IAR expression (Fig.4) are shown together here for the purpose of direct comparison. The same membrane was hybridized with the IA-2 probe, then stripped and rehybridized with the IAR probe as described in the legends to Figs. 3 and 4.

**Figure 6. Purification and phosphatase activity of the intracellular regions of IAR and IA-2.**

**A.** Purified IA-2 (open arrow) and IAR polypeptides (closed arrows) obtained after expression, affinity purification and protease cleavage of GST-fusion proteins. Lane 1, IA-2 obtained from expression of pGEX-3C-IA-2 and cleavage with 3C protease; lane 2, IA-2 obtained from expression of pGEX-KG-IA-2 and cleavage with thrombin; lane 3, IAR obtained from expression of pGEX-3C-IAR and cleavage with 3C protease; lane 4, IAR obtained from expression of pGEX-KG-IAR and cleavage with thrombin. The positions of molecular size markers (kD) are shown to the right of the panel.

**B.** Phosphatase activity of IAR. The IAR was produced as in lane 3 of panel A. IAR was added to a reaction mixture containing 50mM sodium acetate (pH 4.5), 0.5 mg/ml BSA, 0.5mM dithiothreitol and 5mM para-nitrophenyl phosphate (PNPP) and incubated at 30°C. At the times indicated, aliquots of the reaction (each containing 16$\mu$g of purified IAR as measured by Bradford analysis) were removed and mixed with 13% $KHPO_4$ to terminate the reaction. The absorbance of the stopped reaction was monitored at 405 nm and is a measure of the amount of para-nitrophenol product formed by dephosphorylation of PNPP. All reactions were performed in duplicate and the absorbances averaged. Non-enzymatic hydrolysis of PNPP was accounted for by performing appropriate control reactions without added IAR, and this absorbance was subtracted from that attained in the presence of enzyme.

C. Phosphatase activity of IA-2. The IA-2 was produced as in lane 1 of panel A, and phosphatase assays carried out as described above for IAR except that reactions contained 2mM PNPP. Each reaction aliquot removed at the indicated times contained 20$\mu$g of purified IA-2 as measured by Bradford analysis.

**Figure 7. Catalytic activity of IAR.**

A. SDS-PAGE of IAR polypeptide (amino acids 646-1015)

obtained after expression, affinity purification, and proteolytic cleavage of GST-IAR fusion protein. Lane 1, molecular size markers (sizes in kDa shown at left); lane 2, IAR after cleavage with 3C protease. The two uppermost bands in lane 2 are bacterial proteins which are often present after purification of various GST-fusion proteins, including IAR(C945S).

B. pH profile of IAR activity towards PNPP. All reactions were carried out for 30 min and contained 22.3$\mu$g/ml IAR and 2mM PNPP. The buffers used were 50mM sodium acetate (pH 4.0 - 5.0), Mes (pH 5.5 - 6.5) or Tris (pH 7.0 - 8.5).

C. Time-dependent dephosphorylation of PNPP by IAR. Reactions containing 44.4$\mu$g/ml IAR (■) or IAR(C945S) (O) and 5mM pNPP were carried out as described in Materials and Methods and stopped at the times indicated. IAR activity was also measured in the presence of 1mM $Na3VO_4$ (▵). Control reactions lacking IAR were simultaneously performed and the resulting absorbance at 405nm subtracted from that obtained from reactions carried out in the presence of IAR. The data shown are the average of duplicate experiments. Prior to use in the assays described above in B and C, the purified IAR was quantitated by densitometric scanning of Coomassie Blue-stained IAR protein band on SDS-PAGE (indicated by arrow in A) alongside known amounts of protein standards.

**Figure 8. Reactivity of IAR with IDDM sera.**

A. Immunoprecipitation of IAR with sera from control and recent-onset IDDM subjects. *In vitro* translated $^{35}$S-methionine labelled IAR was incubated with sera (prebound to Protein A-Sepharose) from control (subjects 5 and 6 in Table 1) (lanes 1 and 2) and IDDM subjects (subjects 12, 3, and 18 in Table 1) (lanes 3-5). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. The 41 and 32 kDa bands (arrowed) are IAR and an IAR proteolytic product, respectively.

B. Graphical depiction of control and IDDM subject sera

reactivity with IAR. The exact values of the data presented here and further subject information are given in Table 1. Experiments were carried out as in A and the immunoprecipitated IAR was resolved by SDS-PAGE and quantitated (in arbitrary density units) using a phosphorimager.

**Figure 9. Relationship IAR Ab and IA-2 Ab in recent -onset IDDM patients**

Dotted lines represent thresholds of positivity. r=0.70,p<0.0001.

**Figure 10. Effect of IAR Ab (A) and IA-2 Ab (B) on IDDM-free survival in first-degree relatives with ICA ≥ 20JDFU or IAA ≥ 100nU/ml**

Dotted line: antibody-negative subjects, solid line: antibody positive subjects. A:p<0.0001,B: Not Significant.

The invention provides nucleic acids encoding the IDDM-associated autoantigen known as IAR or IAR-PTP and other related polypeptides having the properties of IDDM-associated antigens. Nucleic acids of the invention include the partial IAR-PTP cDNA having the sequence shown in SEQ ID No. 1, the complete cDNA sequence (SEQ ID. No 10) and the cDNA sequence of the cytoplasmic region of the IAR (SEQ ID. No. 12).

The nucleic acids of SEQ ID Nos. 1 and 10 are preferred nucleic acids of the invention. In another preferred embodiment, the nucleic acid of the invention includes a sequence encoding the cytoplasmic domain of IAR, also known as the intracellular domain (SEQ ID No. 12). The DNA sequence encoding the cytoplasmic domain is given in SEQ ID No. 12, along with the encoded amino acid sequence of the cytoplasmic domain (SEQ ID NO. 13). The nucleotide sequence of SEQ ID NO. 12 corresponds to the nucleotides which encode amino acids 646 to 1015 of SEQ ID NO.11. In this embodiment, the sequence of the invention

may consist of the sequence encoding the cytoplasmic domain or it may include other IAR sequences or non-IAR sequences at one or both ends.

However, it will be appreciated that other nucleic acids are included within the scope of the invention.

Nucleic acids of the invention may have substantially the sequence of SEQ ID No. 1, 10 or 12.

Nucleic acids of the invention may consist essentially of the sequence of SEQ ID No. 1, 10 or 12.

The nucleic acid sequences of the present invention are preferably DNA, for example, cDNA or genomic DNA, though they may be RNA. It will be appreciated by those of skill in the art that, in RNA sequences according to the invention, the T residues shown in SEQ ID No. 1, 10 or 12 will be replaced by U. Further, the invention provides both single-stranded and double-stranded nucleic acids.

The nucleic acids of the present invention are not limited to the nucleic acids of SEQ. ID. No. 1, 10 and 12. The nucleic acids of the invention include nucleic acids having sequences that are closely related to these sequences; these nucleic acids encode polypeptides of the invention.

Such nucleic acids of the invention may, for example, be prepared by altering that of SEQ ID No. 1, 10 or 12 by any conventional method, or isolated from any organism or made synthetically. Such alterations, isolations or syntheses may be performed by any suitable method, for example by the methods of Sambrook *et al*: (Molecular Cloning: A Laboratory Manual; 1989).

For example, nucleic acids of the invention may include substitutions, deletions, insertions, or extensions that distinguish them from the nucleic acid of SEQ. ID. No. 1, 10 or 12 as long as these do not destroy the ability of the nucleic acid to encode a polypeptide that functions as an IDDM-associated autoantigen.

A substitution, deletion or insertion may suitably involve one or more nucleotide positions. Thus, nucleic

acid sequences of the invention may differ from those of SEQ ID No. 1, 10 or 12 at any number of nucleotide positions, for example as a result of deletion, substitution, insertion or extension, as long as they encode a polypeptide of the invention. Sequences of the invention may, for example, differ from that of SEQ ID No. 1, 10 or 12 at 1, 2, 3, 4, 5, 6 to 10, 10 to 20, 20 to 30, 30 to 40, 40 to 50 or 50 to 100 nucleotide positions.

For example, the sequences of the invention include sequences that are capable of selective hybridisation to either strand of the sequence of SEQ. ID. No. 1, 10 or 12 and that encode a polypeptide of the invention. Such sequences capable of selectively hybridizing to the DNA of SEQ. ID. No. 1, 10 or 12 will generally be up to 80%, preferably at least 80%, more preferably at least 90%, at least 95% or 99% homologous to the DNA of SEQ. ID. No. 1, 10 or 12. Such homology will preferably apply over a region of at least 20, preferably at least 50, for instance 100, 500 or 1000 or more contiguous nucleotides.

Such hybridisation may be carried out under any suitable conditions known in the art (see Sambrook *et al* (1989): Molecular Cloning: A Laboratory Manual). For example, if high stringency is required, suitable conditions include 0.2 x SSC at 60°C. If lower stringency is required, suitable conditions include 2 x SSC at 60°C.

Also included within the scope of the invention are sequences that differ from those defined above and because of the degeneracy of the genetic code, encode the same polypeptide of the invention. For example, the invention provides degenerate variants of the sequence of SEQ ID No. 1, 10 or 12 that also encode the polypeptide of SEQ ID No. 2, 11 or 13.

The nucleic sequences of the invention may be of any length as long as they encode a polypeptide of the invention. For instance, a nucleic acid sequence according to the invention will typically comprise one or more sequences including one or more of the antigenic epitopes

of IAR-PTP. A nucleic acid sequence according to the invention may be a contiguous fragment of the sequence of SEQ. ID. No. 1, 10 or 12 or a sequence that is related to it in any of the ways described above. Alternatively, nucleic acids of the invention may comprise DNA sequences that are not contiguous in the sequence of SEQ. ID. No. 1, 10 or 12. These sequences may be fragments of the sequence of SEQ. ID. No. 1, 10 or 12 or nucleic acid sequences that are related to such fragments in any of the ways described above. Nucleic acid sequences of the invention will preferably comprise at least 50 bases or base pairs, for example 50 to 100, 100 to 500, 500 to 1000, or 1000 to 2000 bases or base pairs.

In particular, it will be appreciated that SEQ. ID. No. 1 is a partial cDNA sequence of IAR-PTP. The invention also includes sequences extended at either the 3' end or the 5' end, or both ends, by further nucleic acid sequence. 5' extensions are preferred. This sequence may be of any nature and of any suitable length. For example, an extension may comprise up to 10, up to 20, up to 50, up to 100, up to 200, up to 500, up to 1000, up to 1500, up to 2000, up to 2500 or up to 3000 or more bases or base pairs. For example, an extension may comprise the sequence that is contiguous with the sequence of SEQ. ID. No. 1 in the complete IAR-PTP cDNA (SEQ ID. No. 10). Alternatively, it may be any other sequence. Thus, preferred nucleic acids of the invention include complete IAR-PTP cDNAs and fragments thereof which include SEQ. ID. No. 1, particularly human cDNAs and fragments. Also, the invention provides nucleic acids encoding fusion proteins comprising polypeptides of the invention.

A particularly preferred nucleic acid of the invention has the sequence of SEQ ID No. 10, the complete IAR cDNA sequence. Another particularly preferred nucleic acid of the invention has the sequence of the cytoplasmic domain of IAR, SEQ ID NO. 12.

Similarly, the extension or extensions could have any

other sequence. Also, the sequence of SEQ. ID. No. 1, 10 or 12 may be interrupted in one or more places by non-coding sequence. In particular, the non-coding sequence may be introns that occur in the IAR-PTP genomic DNA. Further preferred nucleic acids of the invention are thus IAR-PTP genomic DNAs, and fragments thereof, that include the sequence of SEQ ID No. 1, 10 or 12, particularly human genomic DNAs and fragments thereof.

Three preferred polypeptides of the invention have the sequences shown in SEQ. ID. No. 2, (partial amino acid sequence of IAR), SEQ. ID. No. 11 (complete amino acid sequence of IAR) and 13 (amino acid sequence of cytoplasmic domain of IAR).

SEQ ID No. 13 corresponds to amino acids 646 to 1015 of SEQ ID No. 11. However, the polypeptides of the invention are not limited to the polypeptides of SEQ. ID. No. 2, 11 and 13. Rather, the polypeptides of the invention also include polypeptides with sequences closely related to those of SEQ. ID. No. 2, 11 and 13 that have suitable properties. Thus, the polypeptides of the invention typically have substantially the sequence of SEQ ID No. 2, 11 or 13.

Polypeptides of the invention may have a sequence which consists essentially of the sequence of SEQ ID No. 2, 11 or 13.

Polypeptides of the invention may have a sequence which is substantially the sequence of SEQ. ID. No. 2, 11 or 13.

The polypeptides of the invention are encoded by the nucleic acids of the invention, as described herein. The polypeptides of the invention, IAR-PTP and closely related polypeptides, are IDDM-associated autoantigens, or have the properties of IDDM-related autoantigens.

Thus, the polypeptides of the invention are typically capable of provoking an autoimmune response in individuals who have, or are predisposed to have, or are likely to develop IDDM. Thus, the polypeptides of the invention may

provoke the production of IDDM-associated autoantibodies in such individuals. Also, or alternatively, they may provoke other elements of the autoimmune response.

Further, the polypeptides of the invention are typically recognised by autoantibodies that already present in the sera of such individuals, and are capable of recognising the natural IAR-PTP which contributes to the autoimmune response in these individuals.

Optionally, the polypeptides of the invention may have PTP activity.

Polypeptides of the invention having sequences related to the sequence may be prepared by altering the polypeptide of SEQ. ID. No. 2, 11 or 13 by any conventional method, or isolated from any organism or made synthetically. Such alterations, isolations or syntheses may be performed by any conventional method, for example by the methods of Sambrook *et al* (Molecular cloning: A Laboratory Manual; 1989). In particular, polypeptides related to those of SEQ. ID. No. 2, 11 and 13 may be prepared by modifying DNA sequences as shown in SEQ. ID. No. 1, 10 or 12 and expressing them recombinantly.

Polypeptides of the invention may include substitutions, deletions, insertions, or extensions that distinguish them from the polypeptide of SEQ. ID. No. 2, 11 or 13 as long as these do not destroy the ability of the polypeptide to function as an IDDM-associated autoantigen.

A substitution, deletion or insertion may suitably involve one or more amino acids, typically from one to five, one to ten or one to twenty amino acids. For example, a substitution, deletion or insertion may involve one, two, three, four, five, eight, ten, fifteen, or twenty amino acids. Polypeptides of the invention are substantially homologous to that of SEQ. ID. No. 2, 11 or 13. Typically, a polypeptide of the invention has up to 80%, preferably at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the polypeptide of SEQ. ID. No. 2, 11 or 13.

In general, the physicochemical nature of the polypeptide of SEQ. ID. No. 2, 11 or 13 should be preserved in a sequence of the invention. Such sequences will generally be similar in charge, hydrophobicity and size to that of SEQ. ID. No. 2, 11 or 13. Thus, substitution will typically be conservative. Examples of conservative substitutions that do not greatly affect the physicochemical nature of amino acid sequences are those in which an amino acid from one of the following groups is substituted by a different amino acid from the same group:

H, R and K

I, L, V and M

A, G, S and T

D, E, P and N.

As far as extensions are concerned, a sequence of one or more amino acids may be provided at either or both of the C- and N- termini of the sequence of SEQ. ID. No. 2, 11 or 13 or a sequence related to it in any of the ways defined herein. An extension may comprise up to 5, up to 10, up to 20, up to 50, up to 100, up to 200, up to 300, up to 400, up to 500 or more amino acids. For example, an extension may comprise one, two, three, four, five, up to ten, up to 20, up to 30, up to 50, up to 100, up to 200 or more amino acids.

In particular, extensions may include amino acid sequences which are found in the native complete IAR-PTP protein. Thus, preferred polypeptides of the invention include complete IAR-PTP proteins and polypeptide fragments thereof that include the amino acid sequence of SEQ. ID. No. 2, particularly human proteins and fragments thereof. Also, the invention provides fusion proteins including polypeptides of the invention.

A particularly preferred polypeptide of the invention has the sequence of SEQ. ID. No. 11, the sequence encoded by the complete IAR cDNA (SEQ. ID. No. 10). A further particularly preferred polypeptide has the sequence of SEQ ID NO. 13, which is the sequence of the cytoplasmic domain

of IAR.

A polypeptide of the invention may be subjected to one or more chemical modifications, such as glycosylation, sulphation, COOH-amidation or acylation.

A polypeptide of the invention may comprise multiple copies of the sequence of SEQ. ID. No. 2, 11 or 13 or a sequence related to it in any of the ways defined herein.

A polypeptide of the invention may be of any length as long as it has the ability to function as an IDDM-associated autoantigen. For instance, polypeptides of the invention preferably comprise one or more epitopes of IAR-PTP and therefore retain the ability to provoke an autoimmune response and/or be recognised by preexisting IDDM autoantibodies. Such polypeptides of the invention could be considerably smaller than the polypeptide of SEQ. ID. No. 2.

Polypeptides according to the invention may include one or more fragments of the sequence of SEQ. ID. No. 2, 11 or 13 or fragments that are related to SEQ. ID. No. 2 in any of the ways described above. Such fragments typically comprise at least 10, for example 10 to 20 or 20 to 50, amino acids.

Alternatively, polypeptides of the invention may comprise amino acid sequences that are not contiguous in SEQ. ID. No. 2, 11 or 13. These amino acid sequences may be identical to parts of the native amino acid sequence or related to such parts in any of the ways described herein. For example, polypeptides of the invention may comprise two or more epitopes of IAR-PTP, optionally separated by IAR sequence or non-IAR sequence.

Polypeptides according to the invention preferably comprise at least 10 amino acids, for example 10 to 20, 20 to 50, 50 to 100, 100 to 200, or 200 to 500 or 500 to 1000 amino acids.

Polypeptides and nucleic acids according to the invention may be substantially isolated, isolated, purified or substantially purified. For example, they may be

present in preparations, for example solutions, which have undergone one or more purification steps. Optionally, they may be completely pure. They may be present in preparations, for example solutions, which, apart from the nucleic acid and/or polypeptide of the invention consist essentially of solvents and/or biologically inert carriers. Polypeptides of the invention in substantially purified form will generally comprise the polypeptide in a preparation in which more than 90%, eg. up to 95%, up to 98% or up to 99% of the peptide material in the preparation is that of a polypeptide or polypeptides according to the invention.

The nucleic acids and polypeptides of the invention were originally derived from the human genome. However, nucleic acid sequences and/or polypeptides of the invention may also be obtained from other eukaryotic genomes, especially other mammalian genomes. They may be obtained either by conventional cloning techniques or by probing genomic or cDNA libraries with nucleic acid sequences according to the invention. This can be done by any conventional method, such as the methods of Sambrook *et al* (Molecular Cloning: A Laboratory Manual; 1989). In particular, the complete cDNA and/or genomic DNA sequences of human IAR-PTP can be determined in this way, and the complete amino acid sequence of IAR-PTP can be determined.

A nucleic acid sequence according to the invention may be included within a vector, suitably a replicable vector or an expression vector, for instance a replicable expression vector.

Such an expression vector typically comprises an origin of replication so that the vector can be replicated in a host cell such as a bacterial host cell or a yeast host cell. A suitable vector will also typically comprise the following elements, usually in a 5′ to 3′ arrangement: a promoter for the directing expression of the nucleic acid sequence of the invention and optionally a regulator of the promoter, a translational start codon, a nucleic acid

sequence according to the invention.

The vector may also contain one or more selectable marker genes, for example an ampicillin resistance gene for the identification of bacterial transformants or a marker gene that allows selection of yeast or other eukaryotic transformants. Optionally, the vector may also comprise an enhancer for the promoter. The vector may also comprise a polyadenylation signal operably linked 3' to the nucleic acid of the invention. The vector may also comprise a transcriptional terminator 3' to the sequence encoding the polypeptide of the invention.

The vector may also comprise one or more introns or other coding sequences 3' to the nucleic acid sequence of the invention. The intron or introns may be from the human genome (the organism from which the sequences of the invention were originally derived) or the host organism which is to be transformed with the vector or from another eukaryotic organism.

In an expression vector, the nucleic acid sequence of the invention is operably linked to a promoter capable of expressing the sequence. "Operably linked" refers to a juxtaposition wherein the promoter and the nucleic acid sequence encoding the polypeptide of the invention are in a positional relationship permitting the coding sequence to be expressed under the control of the promoter. Thus, there may be elements such as non-coding sequence 3' or 5' to the coding sequence. These elements may be native either to the human genome or, for example, to the organism from which the promoter sequence is derived.

Alternatively, the said element or elements may be native to neither the organism from which the promoter sequence is derived nor to the human genome. Such sequences can be included in the vector if they enhance or do not impair the correct control of the coding sequence by the promoter.

An expression vector may be of any type. The vector may be in linear or circular form. For example, it may be

a plasmid or viral vector. Those of skill in the art will be able to prepare suitable vectors comprising nucleic acid sequences encoding polypeptides of the invention starting with widely available vectors which will be modified by genetic engineering techniques such as those described by Sambrook *et al* (Molecular Cloning: A Laboratory Manual; 1989).

In an expression vector, any promoter capable of directing expression of a sequence of the invention may be operably linked to the nucleic acid sequence of the invention. For example, suitable promoters include yeast promoters, mammalian promoters, viral promoters and bacterial promoters. Such promoters may be constitutive or regulable. Some suitable promoters include the cytomegalovirus (CMV) early promoter, the SV40 promoter, the mouse mammary tumour virus promoter, the human elongation factor 1 $\alpha$-P promoter (EF-1$\alpha$-P), the SR$\alpha$ promoter and the mouse metallothienein gene I (mMT1) promoter.

Typically, nucleic acid sequences according to the invention will be inserted into such vectors in a sense orientation. However, nucleic acid sequences according to the invention may also be inserted into the vectors described above in an antisense orientation in order to provide for the production antisense RNA. Antisense RNA may also be produced by synthetic means. Such antisense RNA may be used in a method of controlling the levels of the polypeptide of SEQ. ID. No. 2 or 11 or 13 or a protein encoded by a related nucleic acid sequence in a cell. Such a protein is, for example, a complete IAR-PTP protein. Thus, antisense sequences of the invention can form the basis of methods of gene therapy aimed at suppressing the autoantigenicity of IAR-PTP.

Vectors according to the invention may be used *in vitro*, for example for the production of RNA hybridisable to cDNA, of the invention. Such vectors may be used to transfect or transform a host cell. Depending on the type

of vector, they may be used as cloning vectors to amplify DNA sequences according to the invention or to express this DNA in a host cell.

A further embodiment of the invention provides host cells harbouring one or more vectors and/or nucleic acids of the invention. Typically, such cells are transformed or transfected with the vectors for the replication and/or expression of nucleic acid sequences according to the invention, including the DNA of SEQ. ID. No. 1, 10 and 12. The cells will be chosen to be compatible with the vector and may for example be bacterial cells, mammalian, insect or yeast cells.

The cells may be transformed or transfected by any suitable method, such as the methods disclosed by Sambrook *et al* (Molecular Cloning: A Laboratory Manual; 1989). For example, vectors comprising nucleic acid sequences according to the invention may be packaged into infectious viral particles, such as retroviral particles. The constructs may also be introduced by electroporation, calcium phosphate precipitation, biolistic methods or by contacting naked nucleic acid vectors with the cells in solution.

In the said nucleic acid vectors with which the host cells are transformed or transfected, the nucleic acid may be DNA or RNA, preferably DNA, it may be single-stranded or double-stranded.

The vectors with which the host cells are transformed or transfected may be of any suitable type. For example, the vectors may be able to effect integration of nucleic acid sequences of the invention into the host cell genome or they may remain free in the host cell. Typically, the vectors will be expression vectors, such as a retroviral vector or a DNA expression vector as defined herein. For example, the vector used for transformation construct may be a plasmid vector.

The transformed or transfected cells of the invention can be used in a process of production of polypeptides of

the invention. Such processes will typically comprise transforming or transfecting host cells with vectors comprising nucleic acid sequences according to the invention and expressing the nucleic acid sequence in these cells.

In this case, the nucleic acid sequence will be operably linked to a promoter capable of directing its expression in the host cell. Desirably, such a promoter will be a "strong" promoter capable of achieving high levels of expression in the host cell. It may be desirable to overexpress the polypeptide according to the invention in the host cell.

Suitable host cells for this purpose include bacterial cells, for example *E.coli* cells; yeast cells; mammalian cells, for example CHO cells; and insect cells, preferably in combination with a baculovirus expression system. Eukaryotic cells, such as mammalian, insect and yeast cells are preferred in situations where glycosylated polypeptide products are desired.

The thus produced polypeptide of the invention may be recovered by any suitable method known in the art. Optionally, the thus recovered polypeptide may be purified or partly purified by any suitable method, for example a method according to Sambrook *et al* (Molecular Cloning: A Laboratory Manual).

The nucleic acid sequences of the invention may be used to prepare probes and primers. These will be useful in the isolation of nucleic acid sequences having sequences similar to that of SEQ. ID. No. 1, 10 or 12, which may encode polypeptides of the invention. Such probes and primers may be of any suitable length, desirably from 10 to 100, for example from 10 to 20, 20 to 50, 50 to 100 bases in length. Typically, the probes and primers will correspond exactly to part of the sequence of SEQ. ID. No. 1, 10 or 12 over their entire length, although they may vary from this sequence in any of the ways described above. Such probes and/or primers may be used, in particular to

isolate complete IAR-PTP cDNA and/or genomic DNA sequences from the human genome; or from the genomes of other organisms.

The present invention also provides antibodies, preferably monoclonal antibodies, to the polypeptides of the invention. Such antibodies may be useful in methods of diagnosing IDDM, as described herein.

Antibodies of the invention include fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, Fab' and $Fab'_2$ fragments, as well as single chain antibodies.

The antibodies may be produced by any suitable method known in the art, such as the methods of Sambrook *et al* (Molecular Cloning: A Laboratory Manual; 1989). For example, they may be prepared by conventional hybridoma techniques or, in the case of modified antibodies or fragments, by recombinant DNA technology, for example by the expression in a suitable host vector of a DNA construct encoding the modified antibody or fragment operably linked to a promoter, as described above. Suitable host cells include bacterial (for example *E.coli*), yeast, insect and mammalian cells.

Therefore, the invention also provides nucleic acid sequences encoding antibodies of the invention, vectors comprising such sequences, cells harbouring such vectors or sequences, and methods for expressing antibodies of the invention by recombinant techniques.

Polyclonal antibodies may also be raised, using conventional techniques in the art, using the polypeptides of the invention. For example, polyclonal antibodies may be raised by inoculating a host animal, such as a rat, mouse or rabbit, with a polypeptide of the invention, and recovering the immune serum.

Antibodies according to the invention may be useful in methods of the invention, as described herein, for the diagnosis of IDDM.

The invention further provides methods of diagnosing

IDDM. Thus, for example, polypeptides of the invention can be used in methods of diagnosing any aspect of IDDM. For example, they may be used in diagnosing the onset of IDDM, the presence of IDDM, or predisposition to IDDM, or in monitoring the development or progress of IDDM, and in methods of predicting the possible future occurrence of IDDM. For example, individuals who have, or are predisposed to develop, IDDM can be expected to have autoantibodies to the autoantigenic IAR-PTP.

In particular, The IAR polypeptides of the invention are useful in methods carried out on individuals having a high likelihood of developing IDDM, for example, relations, especially close relations, more especially first degree relations (parents and siblings), of known IDDM sufferers.

For example, the IAR polypeptides of the invention are particularly useful in determining the likelihood that a close relation of an IDDM sufferer will contract IDDM. Close relations include, for example, parents, grandparents, siblings, children, grandchildren, cousins, uncles, aunts, nieces, grand aunts and uncles and grand nieces and nephews.

Thus, samples of biological fluid suspected of containing such autoantibodies can be taken from individuals and contacted with polypeptides of the invention. The anti-IAR autoantibodies, if present, will recognise the polypeptides of the invention and this recognition can be detected, for example by detecting the IAR/anti-IAR complex thus formed.

Thus, the methods of the invention typically comprise a step of determining whether or not recognition of the polypeptide of the invention by autoantibodies occurs in the future. Similarly, if no such recognition occurs, the test suggests that the individual does not have IDDM or is unlikely to develop it in the future.

Recognition of polypeptides of the invention by antibodies present in such a sample correlates with IDDM or a potential for developing IDDM.

The methods of the invention are thus typically methods of assaying for the presence or absence of autoantibodies that recognise IAR. They may also quantitate the amount or concentration of such autoantibodies in a sample.

Thus, diagnostic methods of the invention typically comprise:

(a) contacting a sample from an individual with a polypeptide according to the invention under conditions that permit the recognition of the polypeptide by autoantibodies that recognise IAR; and

(b) determining whether or not said recognition occurs.

Any suitable sample from the individual may be used. Typically the sample will comprise a biological fluid, preferably blood, plasma or serum. The sample may be of any size.

The sample is contacted with a polypeptide of the invention and the presence or absence of immunoreactivity between them is determined.

In some embodiments of the invention, a second IDDM-related autoantigen may be used. Thus, the invention provides methods, as described above, which further comprise, in addition to steps (a) and (b), contacting the sample with at least one further IDDM-related autoantigen, preferably an islet cell antigen (ICA). This may be done at the same time, or at a different time to the contacting with the polypeptide of the invention.

In such methods, it is determined whether or not autoantigens recognise the further autoantigen or autoantigens. The same sample or a different sample from the same individual may be contacted with the polypeptide of the invention and the further IDDM-associated autoantigen. Such a further autoantigen may be, for example, hIA-2, GAD, ICA-512, ICA-12, ICA-13, ICA-208, ICA-302, ICA-313, ICA-525 or ICA-505; or a polypeptide that functions as one of these further autoantigens.

Preferred further autoantigens include: IA-2,

preferably hIA-2; ICA-512; and GAD; and antigenic fragments thereof. Preferred combinations of autoantigens therefore include: a polypeptide of the invention and one, two or all three of IA-2, GAD and ICA-512. Further IDDM-associated autoantigens may be used, as appropriate, in any of the techniques described below.

Any conventional technique may be used to determine whether or not polypeptides of the invention are recognised by autoantibodies. This also applies to determining whether or not any further autoantigens which may be used are recognised by autoantibodies.

For example, suitable immunoassay formats employ a combination of solid phase or immobilized reagents and labelled reagents whereby the association of the label with the solid phase is a function of the presence or absence of recognition of the polypeptide of the invention or a further autoantigen. In general, such a solid phase reagent comprises a binding substance such as a polypeptide of the invention, an anti-antibody (e.g., anti-IgG), or other immunobinder or other binding agent according to the assay protocol involved, bound or attached, covalently or non-covalently, to a solid phase matrix or in an otherwise immobilized form. Suitable solid phase matrices are conventional in the art and include such matrices as microtiter plate wells, test tubes and other test containers or vessels, test strips, beads, and particles such as plastic microparticles and latexes. Where a solid phase reagent comprises more than one type of polypeptide of the present invention and/or a polypeptide of the invention and one or more further autoantigens, it will be recognised that each reagent can be physically separated or isolable from the others or two or more can be mixed or otherwise associated in an undifferentiated manner with the solid phase. For example, reagents can be immobilised in separate wells of a microtiter plate or can occupy the same wells; or, where the solid phase is in the form of particles, each individual particle can have attached only

a single reagent or can have attached two or more reagents.

Similarly, useful labelled reagents comprise a binding substance such as a polypeptide of the invention, a further autoantigen, an anti-antibody (e.g., anti-IgG), or other immunobinder or other binding agent according to the assay protocol involved, which is chemically coupled with a detectable chemical moiety. Useful labels are conventional in the art and include fluorescers, chemiluminescers, radioisotopes, and enzymes. Enzyme labels are particularly useful and are generally selected from alkaline phosphatase, peroxidase, and $\beta$-galactosidase. Enzyme labels are readily detectable by addition of a corresponding chromogenic substrate and detecting the resulting colour or fluorescent response.

One particular immunoassay format that can be applied to the present method employs an immobilized form of the polypeptides of the invention, and optionally an immobilized form of a further autoantigen, as an immunoreagent. A test sample is incubated with the solid phase reagent and preferentially washed to remove unbound material. A labelled antibody reagent is then added. Such antibody reagents can be specific for a particular class of immunoglobulin, e.g., IgG, IgM, IgA, etc., or can be a mixture of conjugates so that all immunoglobulin types are detectable. Islet cell antibodies of interest are generally of the IgG isotype and thus anti-IgG would normally be employed. The solid phase is washed to remove unbound labelled antibody reagent and the label activity remaining on the solid phase is measured qualitatively or quantitatively.

A variation of this protocol uses a ligand-modified form of the polypeptide of the invention, and optionally a ligand-modified form of a further autoantigen with immobilization to the solid phase being accomplished by using a solid phase bearing a binding partner to the ligand. For example, biotin or a hapten (e.g., fluroscein) can be used as the ligand and can be rendered immobilized

by contact with a solid phase form of avidin or anti-hapten antibody, respectively. The addition of the solid phase binding partner can occur at any convenient time in the assay, such as prior to contact of sample with the ligand-antigen(s) or thereafter.

Another immunoassay format that can be applied to the present method employs an immobilized form of an antibody reagent. Antibody specific for the desired ICA immunoglobulin type to be detected (e.g., IgG), or a mixture of antibodies against different IgG isotypes, is immobilized on a solid phase and incubated with the test sample. Resulting islet cell antibody that has become bound to the solid phase antibody reagent can then be detected in any suitable manner. For instance, one can add labelled forms of one or more types of polypeptide of the invention and/or of other IDDM autoantigens either as individually labelled reagents or as a combined labelled reagent. Alternatively, one can add a soluble form of the polypeptide of the invention (and optionally a further autoantigen) and then, together in the form of a complex, or later as a separate reagent addition, labelled antibody that can bind with it. Further, one can add a soluble form of the polypeptide of the invention (and optionally a further autoantigen) which has been modified with a ligand and then adding a labelled form of a binding partner to the ligand. The previously described variation of using a ligand-modified form of the solid phase reagent, in this case, antibody, with immobilization to the solid phase being accomplished by using a solid phase bearing a binding partner to the ligand, can also be used.

A competitive immunoassay format is also useful. Immobilized polypeptide of the invention is employed along with a labelled form of islet cell antibodies, for example antibodies of the invention as described herein. Labelled antibody and ICA from the test sample are allowed to compete for binding to the polypeptide of the invention either simultaneously or sequentially, e.g, by exposing the

solid phase polypeptide of the invention first to the sample and thereafter to the labelled antibody reagent. The polypeptide of the invention can be immobilized directly to the solid phase or through a ligand-binding partner bridge as described above, with the immobilization step being performed at any convenient time in the assay, including as the last step.

Latex or particle agglutination methods are also suitable. Particles are coated or covalently coupled with the polypeptides of the invention, and optionally with one or more further IDDM-associated autoantigens. The particles are then incubated with the test sample and resulting agglutination of the particles due to the formation of ICA-antibody linkages between particles is detected. Detection can be accomplished, for example, by visual observation (a slide agglutination format) or quantitated by measuring turbidity change with a spectrophotometer or nephelometer. A variation is based on inhibition of particle agglutination. Each particle reagent comprises one or more monoclonal antibodies corresponding specifically with one or more particular polypeptide of the invention and optionally with one or more further IDDM-associated autoantigens, respectively. In addition, an agglutinator reagent is prepared comprising multiple antigens, e.g., a water soluble polymer backbone to which are attached multiples of one or more types of polypeptides of the invention, and optionally one or more further autoantigens. When mixed with sample, the absence of autoantibodies in the sample results in agglutination of the particles by formation of bridges formed by the agglutinator reagent. When autoantibodies are present, they bind to the agglutinator and thereby prevents or inhibits agglutination.

In methods according to the invention, any suitable quantity of polypeptides of the invention and/or of the further autoantigens may be used. For example, for each test according to the invention, a quantity of from

$1 \times 10^{-12}$g to $1 \times 10^{-6}$ g of a polypeptide of the invention and optionally of one or more further autoantigens as defined herein may be used. Preferably, quantities of from $1 \times 10^{-10}$g to $1 \times 10^{-7}$g may be used, with quantities of from $10^{-9}$g to $10^{-7}$g being more preferred. For example, quantities of from 10 to 50 ng, for example from 20 ng to 30ng may be used ($1 \times 10^{-8}$g to $5 \times 10^{-8}$g). Such quantities of polypeptides of the invention and optionally of further autoantigens as defined herein, may, for example, be applied to a given microtiter well in a microtiter plate or to any other solid phase matrix.

Diagnostic methods of the invention are typically performed *in vitro* or *ex vivo* although, where appropriate, *in vivo* methods are contemplated.

The methods of the invention have a number of clinical applications related to IDDM. For example, the methods of the invention are useful in screening of patients from the general population, particularly juveniles, wherein a positive test result (i.e., a finding that a patient's blood contains antibodies that bind to a polypeptide of the invention) indicates that such a patient is at risk of developing IDDM, that is, that such a patient may have a pre-IDDM condition. Furthermore, the present method is applicable to the screening of first degree relatives (i.e., siblings and children) of individuals already diagnosed as having IDDM. Patients with positive test results from the methods of the invention could be examined in more detail, for example by means of metabolic and genetic testing, to determine whether glucose intolerance has already occurred, or whether additional risk factors are present.

Other applications of the present method include the testing of a patient already diagnosed with diabetes for the purpose of determining if IDDM is involved, for instance in the case of a Type II diabetic who may also experience the onset of IDDM.

Furthermore, the methods of the invention are useful

in monitoring of diabetic therapy. For example, using the methods of the invention, it is possible to monitor the effectiveness of any type of treatment of IDDM, for example gene therapy, immunosuppressant therapy or immune therapy. it is also possible to monitor the effects of drug therapy, the impact of diet on IDDM, and the effect of other factors on IDDM.

The invention further provides medical uses of the polypeptides of the invention. Accordingly, the invention provides polypeptides of the invention for use in methods of treatment of the human or animal body, especially the treatment of IDDM; and for use in methods of diagnosis of IDDM. In methods of treatment of IDDM, polypeptides of the invention may, for instance, be used to saturate an individual's immune response, blocking the individual's autoimmune response to natural IAR-PTP. The invention also provides polypeptides of the invention for use in methods of treatment of the human or animal body, particularly for IDDM, in combination with one or more further autoantigens as described herein.

Further, the invention provides the use of polypeptides of the invention in the manufacture of medicaments for the treatment of the human or animal body, especially the treatment of IDDM; and for use in methods of diagnosis of IDDM, as described herein. Such medicaments may comprise a further IDDM-associated autoantigens as described herein, or may be administered in combination with such a further autoantigen.

Similarly, the invention provides pharmaceutical compositions comprising a polypeptide of the invention, and optionally a further autoantigen as defined herein; and a pharmaceutically acceptable carrier.

The invention further provides diagnostic test kits comprising polypeptides and/or antibodies of the invention, optionally together with one or more further IDDM-associated autoantigens, as described herein.

Any suitable quantity of these components may be

present in the test kits. Other components may also be included in the kits, for example buffers and/or other solutions; labels; tubes, plates, membranes, membrane sticks or other items of equipment, for example for immobilising reagents; and/or anti-human antibodies for monitoring immunoreactivity.

Test kits of the invention typically further comprise means for determining whether or not the polypeptides of the invention are recognised by autoantibodies. Test kits comprising one or more further autoantigens typically include means for determining whether or not the further autoantigen or autoantigens are recognised by autoantibodies.

Test kits according to the invention may, for example, enable the use of methods according to the invention. Thus, they may comprise, as means for determining whether or not the polypeptides of the invention, and optionally one or more further IDDM-associated autoantigens as described herein, are recognised by autoantibodies, any reagents suitable for putting into practice the techniques described above.

In test kits of the invention, the components may be in any suitable form; for example they may be packaged in any suitable container. The components may be intended for separate, for example sequential, use; or for concurrent use.

In test kits of the invention, the components may be in any suitable form, for example they may be in sterile solutions, buffered solutions or sterile and buffered solutions. They may be labelled or unlabelled. The components may be in substantially purified, purified, isolated or substantially isolated form.

The following Examples illustrate the invention.

**EXAMPLES**

**1. Initial identification of IAR as a PTPase**

Primers corresponding to conserved amino acid

sequences (DYINA (SEQ. ID. No. 14) and VHCSAGV (SEQ. ID. No. 15) of the catalytic domain of PTPases were designed (with additional EcoRI sites added) and used to amplify a human colon carcinoma cell (SW480) cDNA library (Clontech, HL3014b) using PCR. The primers used had the following sequences: 5'-GGGAATTCNGAYTAYATHAAYGC-3' (SEQ ID No. 3) and 5'-GGGAATTCACNCCNGCRCTRCARTGNAC-3' (SEQ ID No. 4). The PCR was carried out as follows: 94°C x 5 min --->(94°C x 40 sec--->50°C x 30 sec--->72°C x 90 sec) x 30--->72°C x 5 min--->4°C. The major amplified fragment of ~460bp was subcloned into the pGEM-T vector (Promega) and 94 clones were sequenced.

One clone contained an amplified fragment which did not have high homology to any known PTPase. This fragment was selected for further study and was termed r75. r75 was identical over a 312bp portion of its sequence to a human brain EST sequence (# 03250) [Adams *et al.*, 1993, Nature Genet. 4, 256-267] which was noted in this paper as having 80.6% similarity to ICA512).

To examine the size and tissue expression of RNA transcripts from which r75 originated, human multiple Northern blots (Clontech) were probed with $^{32}$P-labelled r75 (Fig. 1). Two major transcripts of about 3.7kb and 5.5kb were observed. The highest levels of expression were detected in brain and pancreas, followed by prostate and testis, with lower expression detectable in a few other tissues, including colon.

## 2. Cloning of IAR

Based on the expression pattern of transcripts recognized by r75, a random primed human pancreas cDNA library in λgt11 (Clontech, HL1163b) was screened using r75 as probe. The r75 was labelled with $^{32}$P-dCTP (Amersham) using the High Prime Labeling Kit (Boehringer Mannheim) and denatured by heating before hybridization. Hybridization was carried out overnight at 65°C in 0.5M sodium phosphate (pH 6.8), 7% SDS, 15% formamide, 1mM EDTA. The membrane

was washed 3 times at 65°C with 50mM sodium phosphate (pH 6.8), 0.1% SDS, and exposed to film at -70°C. Positive clones were reselected through another 3 rounds of subscreening with r75. Continuously positive clones were subcloned into the EcoRI site of Bluescript SK(+) (Stratagene) and partially sequenced for comparison with r75. Some clones were found to be fragments of IA-2 cDNA (Lan *et al.*, 1994). One of these clones, D2, contained nucleotides 1589-3166 of IA-2. Several other clones were distinct from IA-2. Of these, the largest clone (C3) containing an open reading frame of about 2.5kb was selected for sequencing. The C3 clone was digested by PstI or ApaI into shorter fragments. Each fragment was subcloned into Bluescript SK(+) and sequenced across both strands using either primers to the vector or synthesized primers hybridizing to the insert in conjunction with the Sequenase Version 2.0 Sequencing Kit (United States Biochemical Corporation).

The C3 clone contained a coding sequence missing initiation and termination signals, and was significantly smaller than than the 3.7 and 5.5kb RNA transcripts detected by Northern blotting with r75 (Fig.1), suggesting that it was incomplete at both the 5' and 3' ends. The 3' end of the clone was obtained by 3' RACE using the Marathon cDNA Amplification Kit (Clontech). The first-strand cDNA was reverse transcribed from human brain polyA$^+$ mRNA (Clontech) using the cDNA synthesis primer provided with the kit. The gene-specific primer used for 3'-RACE was 5'-CCTGCCTCCTCAGGCGGAGCAAGA-3' (SEQ ID No. 9), corresponding to nucleotides 1389-1412 of SEQ ID No.1. PCR was carried out at 94°C x 3 min--->(94°C x 40 sec--->68°C x 4 min) x 30--->4°C using the Expand Long PCR Kit (Boehringer Mannheim). The amplified fragments were cloned into the pGEM-T vector (Promega) and partially sequenced for comparison with C3. One clone, 3-7, with a region of identical overlap with C3, contained a stop codon and polyadenylation signal and was sequenced along both strands by Sanger's dideoxy-mediated

chain-termination method. Another clone, 3-10, was about 2kb larger than 3-7. The 3-10 clone was identical to 3-7 from the 5' end up to the polyadenylation signal in 3-7, but was followed by approximately 2kb of additional 3' untranslated sequence.

The combined sequences of C3 and 3-7 comprise a partial cDNA encoding a novel putative receptor-like PTPase which we term IAR (for islet cell antigen-related) (Fig.2). The predicted protein fragment (SEQ. ID. No.2) translated by the only possible open reading frame comprises an incomplete N-terminal extracellular region, a 21 amino acid transmembrane-spanning region, and a 379 amino acid intracellular region.

To obtain the 5' end of the cDNA, the pancreas library was rescreened with a 300 bp probe close to the 5' end of C3. No IA-2 clones were found. Of the positive clones, clone B11 appeared to have the longest 5' extension to C3 and was selected for further sequencing along both strands. The 3' end of B11 was identical to C3 sequence over a length of about 2 kb, but the 5' ends of B11 and C3 were different (Fig. 2C). B11 had a unique 5' sequence of 412 bp, followed by a 54 bp sequence that is found in the opposite orientation in C3. The sequence of C3 found 5' to this inverted region was shorter and different from that of B11. 5' RACE was used to determine which clone contained the correct 5' sequence and to try to obtain additional 5' sequence to the open reading frames of B11 and C3. Template cDNA was prepared from human brain polyA$^+$ mRNA (using the Marathon cDNA amplification Kit (Clontech) using random primers or a primer (P5, with the sequence 5'-CGTGTGGGCCACATAGGTCAGGATGCTCTCGGAGAA-3' (SEQ. ID. No. 20)) corresponding to shared B11/C3 sequence located about 170 bp 3' to the invert. The 5' RACE used a primer to the ligated adaptor sequence at the ends of the cDNA and an IAR specific reverse primer (P11 with the sequence 5'-TGGCGAGCACGTCTGAGGCTG-3' (SEQ. ID. No. 21)) to a region of shared B11/C3 sequence located about 90 bp 3' to the

invert. The amplified fragments were cloned into pGEM-T (Promega) and sequenced along both strands. Cloning and sequencing of the 5' RACE PCR products from both types of template cDNA showed that all had B11 sequence. Similarly, PCR amplification of the pancreas cDNA library using a primer to λgt11 and either P5 or P11 primers gave DNA fragments which all corresponded to the unique B11 sequence. Thus the B11 sequence is correct and the 5' end of the C3 clone is likely a library artifact. No PCR products were found with a 5' extension of the B11 sequence, and no 5'-extended clones were identified by further screening of human brain and pancreas cDNA libraries.

Although no in-frame stop codons were found 5' to the first ATG codon in the B11 sequence, we propose that this is the initiation codon for the following reasons: 1) a purine (G) in the -3 position conforms with the Kozak rules of initiation (Kozak (1989)), and 2) this methionine is followed by a 20 residue hydrophobic sequence that has the features of a signal sequence (Von Heijne 1986)). To test for signal sequence function, cDNA encoding full-length IAR protein was transcribed and translated *in vitro* in the presence of microsomal membranes. Subsequent treatment with Proteinase K reduced the size of the translated product to a fragment corresponding to the predicted size of the extracellular region of IAR (data not shown). This suggests that the IAR protein is inserted into the membranes such that the extracellular region of IAR is in the Proteinase K-inaccessible interior of the membrane vesicles.

The complete cDNA (SEQ. ID. No. 10) is predicted to encode a receptor-like PTP of 1015 amino acids (SEQ. ID. No. 11). This includes a 614 amino acid extracellular region, a 25 residue hydrophobic transmembrane segment, and a 376 amino acid intracellular region containing a single PTP catalytic domain (SEQ. ID. No. 11). This protein has about 43% overall sequence identity to the putative PTP IA-

2 (Lan *et al* (1994); Rabin *et al* (1994)), a protein identified as an islet cell auto-antigen in IDDM, and another PTP-like molecule called PTPLP ((Kambayashi *et al* (1995)). We have thus named the novel PTP as islet cell antigen-related PTP or IAR PTP. The IAR extracellular region does not contain FN-III or Ig-like repeats, but has the adhesion recognition peptide sequence RDGS (amino acids 372-375). There is one potential site for N-linked glycosylation. The catalytic domain of IAR has conserved sequences typical of other PTPases, although the active site is unusual in having an aspartate residue at position 947 (IVHCSDGAGRTG (SEQ. ID. No. 16)) in place of a conserved alanine residue.

In view of the homology of IAR and IA-2, we selected non-homologous fragments of the C3 and D2 clones to use as specific probes on Northern blots in order to determine whether distinct transcripts were identified. The IAR probe comprised nucleotides 790-1197 of SEQ. ID. No.1 (corresponding to a part of the predicted extracellular region), and the IA-2 probe comprised nucleotides 1665-1906 of IA-2 cDNA (Lan *et al.*, 1994), corresponding to a part of the extracellular region, the transmembrane region, and a small portion of the intracellular region. These probes were labelled with $^{32}P$ as described in the legends to Figs. 3 and 4. A set of human multiple tissue Northern blots (Clontech) was probed first with the IA-2 probe (Fig.3), and later stripped and reprobed with the IAR probe (Fig. 4). The two probes recognize distinct transcripts in terms of size and tissue specificity. The IA-2 probe detects a single transcript of about 3.9kb (Lan *et al.*, 1994), whereas the IAR probe detects two transcripts of about 3.7 and 5.5kb. It is possible that the 3.7kb transcript has a 3′ untranslated sequence corresponding to that of the 3-7 clone, while the larger 5.5kb transcript represents an alternative 3′ untranslated sequence corresponding to that in the longer 3-10 clone. The highest expression of IA-2 is detected in brain, followed by spinal cord and pancreas,

with low levels expressed in small intestine and adrenal gland. The highest expression of IAR is in pancreas and brain, followed by trachea, prostate, stomach and spinal cord, with low levels detectable in small intestine and adrenal gland. Figure 5 shows an alignment of the IAR and IA-2 transcripts detected in brain and pancreas. It is obvious that the IA-2 transcript is larger than the smallest 3.7kb IAR transcript, and furthermore the 3.7kb IAR transcript is much more highly expressed in pancreas than brain while the 3.9kb IA-2 transcript is more highly expressed in brain than pancreas. Thus IAR and IA-2 appear to be encoded by distinct genes.

**3. Expression and analysis of the intracellular region of IAR**

The intracellular region of IAR was amplified by PCR using the 3-7 clone as template. The primers used had the sequences 5'-GGGCTCGAGTCTAGACAGGCTGAAGGAGAAGCTCTC-3' (SEQ ID No. 5) (corresponding to nucleotides 1517-1538 of SEQ. ID. No.1, with added XbaI and XhoI sites) and 5'-GGGGAATTCCATGGTTATAATAGAAGACACACA-3'(SEQ ID No. 6) (corresponding to nucleotides 2704-2723 of SEQ. ID. No.1, with added EcoRI and NcoI sites). PCR was carried out at 94°C x 5 min--->(94°C x 40 sec--->60°C x 30 sec--->72°C x 2 min) x 10--->72°C x 5 min--->4°C using the Expand Long PCR Kit (Boehringer Mannheim). The amplified fragment was cloned into the XbaI and NcoI sites of pGEX-KG (Guan and Dixon, 1991) and sequencing of the entire insert confirmed that no mutations had resulted from PCR. Later, the insert was cut out of this plasmid with XhoI and EcoRI and cloned into these sites in pGEX-3C (Walker *et al.*, 1994). Mutant IAR (C945S) was generated by PCR using overlapping forward and reverse primers corresponding to the desired mutation and surrounding sequence encoding IIVHSSDGA (SEQ. ID. No. 19); 5'-ATAATTGTTCATTCCAGTGACGGTGCA-3' (SEQ. ID. No. 17) and 5'-TGCACCGTCACTGGAATGAACAATTAT-3'(SEQ. ID. No. 18). Each

primer was used in combination with one of the primers (of SEQ. ID. No. 5 or 6) (used to amplify the intracellular region of IAR) to generate two overlapping DNA fragments corresponding to nucleotides 1993-2904 and 2878-3197 of IAR. These fragments were mixed and used as template for a PCR reaction with the primers of SEQ. ID. No. 5 and 6. The above plasmids were transformed into *E.coli* (DH5$\alpha$F'). The recombinant bacterial host was cultured and GST-IAR expression induced with 0.15mM IPTG. The GST-IAR was purified from bacterial lysates by standard methods (Smith and Johnson, 1988). The IAR was cleaved from GST using thrombin or 3C protease as appropriate (Smith and Johnson, 1988; Walker *et al.*, 1994), and the purified IAR quantitated by SDS-PAGE followed by densitometric scanning alongside known amounts of standards as described (Wang and Pallen, 1991). Cleavage with thrombin of GST-IAR produced from the pGEX-KG plasmid resulted in a major protein band on SDS-PAGE which migrated with an apparent size of about 36kD (Fig. 6A, lane 4). This was smaller than the predicted size of the expressed IAR insert, suggesting that internal cleavage of the IAR polypeptide by thrombin might have occurred. This led to recloning of the insert into the pGEX-3C vector as described above. Cleavage with 3C protease of GST-IAR produced from this plasmid resulted in a major protein band on SDS-PAGE which migrated with the expected size of about 40kD (Fig.6A, lane 3).

The purified 40kD form of the IAR intracellular region was analyzed for phosphatase activity. Low but detectable phosphatase activity towards the substrate para-nitrophenyl phosphate was measured (Fig. 6B). Since the related molecule IA-2 reportedly has no detectable phosphatase activity (Rabin *et al.*, 1994; Lu *et al.*, 1994), we examined this in side-by-side experiments with IAR.

**4. Expression and analysis of the intracellular region of IA-2**

The intracellular region of IA-2 was amplified by PCR

using the D2 clone as template. The primers used had the sequences 5'-GGGCTCGAGTCTAGAGCGGCAGCAAGACAAGGAG-3' (SEQ ID No. 7) (corresponding to nucleotides 1885-1903 of IA-2 (Lan *et al.*, 1994), with added XbaI and XhoI sites) and 5'-GGGGAATTCGAGCTCGCATGCCCAAGAGGTGGC-3' (SEQ ID No. 8) (corresponding to nucleotides 3104-3121 of IA-2, with added EcoRI and SacI sites). PCR was carried out as described above for the IAR intracellular region. The amplified fragment was cloned into the XbaI and SacI sites of pGEX-KG (Guan and Dixon, 1991). Later, the insert was cut out of this plasmid with XhoI and EcoRI and cloned into these sites in pGEX-3C (Walker *et al.*, 1994).

Recombinant bacterial GST-IA-2 protein was produced from both the above plasmids, cleaved, purified and quantitated as described above for IAR. However, unlike IAR, the type of protease used for cleavage from GST did not affect the size of the IA-2 produced, with the purified IA-2 migrating on SDS-PAGE in both cases as a major band with an apparent size of 44kD (Fig.6A, lanes 1 and 2). Thus the IAR and IA-2 proteins differ in that IAR appears to possess an internal thrombin cleavage site which is absent in IA-2.

**5. Catalytic activity of IAR**

The purified 44kD form of IA-2 (produced by cleavage with 3C protease) possessed phosphatase activity towards para-nitrophenyl phosphate which was roughly comparable to that of IAR (Fig. 6C).

The intracellular region of IAR (amino acids 646-1015) was expressed as a GST-fusion protein and purified following 3C protease cleavage from GST. The IAR migrated on SDS-PAGE as a major protein band of about 41 kDa (Fig. 7A, lane 2), in accord with the predicted size of 41.7 kDa. The purified IAR possessed low but detectable phosphatase activity towards pNPP. The IAR is active over a narrow pH range, with optimal activity at pH 4.5, and is essentially inactive at pH 5.5 (Fig.7B). IAR catalyzes the time-

dependent dephosphorylation of pNPP (Fig. 7C) with a specific activity of 21 nmol/min/mg. Like many PTPs, IAR activity is sensitive to inhibition by vanadate, and 1mM sodium orthovanadate completely abolished activity (Fig. 7C). Site-directed mutagenesis of the essential cysteine residue (to a serine) in the active site resulted in the expression of an IAR intracellular region polypeptide which did not catalyze pNPP hydrolysis (Fig. 7C), indicating that the phosphatase activity measured with the wild type polypeptide was unlikely to be due to contaminants in the protein preparation. IAR dephosphorylated phosphotyrosyl casein but not phosphoseryl casein.

**6. Reactivity of IAR with IDDM Patient Sera**

**Patients and Sera.** Sera were obtained from healthy blood bank controls (n=10) and from recent-onset IDDM patients (n=20, age 4.0-15.7 years) prior to commencement of insulin therapy. ICA were measured by indirect immunofluorescence on frozen sections of human pancreas (Bonifacio *et al* (1990)), and GAD antibodies were measured by enzymatic immunoprecipitation (Schmidli *et al* (1994)).

***In Vitro* Translation and Immunoprecipitation Tests with Patient Sera.** cDNAs encoding amino acids 646-1015 of IAR (representing the intracellular region) or full-length IA-2 protein (Lan et al (1994)) were subcloned into the pGEM-3 vector, under the control of the SP-6 promoter. Radiolabelled IAR and IA-2 proteins were synthesized by *in vitro* translation and transcription in the presence of $^{35}$S-methionine, using a reticulocyte lysate system (Promega TNT Kit). For immunoprecipitations, 1$\mu$l of lysate containing labelled IAR or IA-2 in 100$\mu$l TBST-BSA (50mM Tris-HCl, 150mM NaCl, 1% Tween-20, 0.1% BSA) was incubated overnight at 4 °C with 2$\mu$l of serum which had been prebound to 10$\mu$l (packed bead volume) Protein A-Sepharose (Pharmacia). Beads were washed once with TBST-BSA, once with high salt wash buffer (0.5% Triton X-100, 200mM NaCl, 50mM $NaH_2PO_4$),

twice in PBS plus 0.5% Triton X-100 and once in 0.5% Triton X-100, 4.17mM Tris, 192mM glycine, pH 7.4. Precipitated proteins were resolved by 10% SDS-PAGE. Gels were dried and exposed to a phosphorimager, and bands were quantified by densitometry after subtraction of local background. Sera from 10 healthy blood bank control and 20 recent-onset IDDM subjects (ICA, GAD antibodies, age and sex of subjects are shown in Table 1) were incubated with *in vitro* translated $^{35}$S-methionine labelled IAR (amino acids 646-1015). Immunoprecipitates were analyzed by SDS-PAGE, where the translated IAR protein appeared as a band of 41 kDa with an associated proteolytic breakdown product of 32 kDa. Representative immunoprecipitations of IDDM and control sera are shown in Fig. 8A. Quantitative analysis of a panel of sera showed that band densities were greater than the mean +2SD of the 10 control sera (1818 density units) in 11 of the 20 (55%) recent-onset IDDM sera (Table 1). There was no significant correlation, in this small number of subjects, between IAR and ICA, GAD antibodies, or age. The IAR reactivity of the control and IDDM subjects is shown graphically in Fig. 8B. Sera from 9 of the 11 IAR-reactive patients also immunoprecipitated IA-2, some reacting more strongly with IAR than IA-2 and the inverse seen with others (Table 1). Of interest is the finding that 3 IDDM subjects had antibodies which immunoprecipitated IAR or IA-2, but not both (Table 1, subjects 4, 13, 20). Thus IAR and IA-2 can distinguish different autoantibody populations in a subset of IDDM patients.

Table 1. Reactivity of sera with ICA, GAD, IAR and IA-2

| Patient | Age | Sex | ICA | GAD | IAR | IA-2 |
|---|---|---|---|---|---|---|
| Control | | | | | -1039 | -1198 |
| 2 | | | | | -8 | 366 |
| 3 | | | | | 734 | -350 |
| 4 | | | | | 1611 | 122 |
| 5 | | | | | 58 | -2665 |
| 6 | | | | | 412 | -129 |
| 7 | | | | | 642 | 748 |
| 8 | | | | | 388 | 669 |
| 9 | | | | | 756 | -62 |
| 10 | | | | | 828 | -2894 |
| IDDM 1 | 12.1 | F | 0 | 209 | 1092 | 508 |
| 2 | 5.5 | M | 160 | 522 | 1084 | -1326 |
| 3 | 6.0 | M | 140 | 180 | 312286 | 13491 |
| 4 | 13.1 | F | 42 | 272 | 7963 | 318 |
| 5 | NA | NA | NA | NA | 2148 | 12990 |
| 6 | 4.0 | F | 270 | 3291 | 365 | 170 |
| 7 | 12.9 | F | 0 | 238 | 428 | 691 |
| 8 | 13.4 | M | 84 | 184 | 376 | 470 |
| 9 | 12.8 | F | 270 | 3335 | 21802 | 18732 |
| 10 | 10.7 | M | 63 | 283 | 5392 | 5626 |
| 11 | 12.5 | F | 32 | 1713 | 631 | -567 |
| 12 | NA | NA | NA | NA | 150105 | 16493 |
| 13 | 9.8 | F | 120 | 839 | 4344 | -140 |
| 14 | 10.5 | M | 270 | 167 | 17107 | 37610 |
| 15 | 3.8 | F | 270 | 483 | 5564 | 10186 |
| 16 | 7.0 | M | 270 | 199 | 160 | -1365 |
| 17 | NA | NA | NA | NA | 19517 | 11265 |
| 18 | 4.6 | M | 270 | 126 | 21006 | 12713 |
| 19 | 9.6 | M | 26 | 235 | 1232 | 1726 |
| 20 | 15.7 | F | 160 | 1959 | -378 | 4717 |

**8. Comparison of the predictive value of IAR and IA-2 antibodies for IDDM (see Figures 9 and 10)**

Sera from recent-onset IDDM subjects (n=53) were

obtained piror to commencement of insulin therapy. The healthy control group (n=144) consisted of laboratory volunteers and healthy schoolchildren. First-degree relatives of subjects with IDDM (n=3315) were enrolled in the Melbourne Prediabetes Family Study and had ICA and IAA measured at entry. These subjects form an "at-risk" group in that the risk of developing IDDM in ICA positive relatives has been previously shown to be 35% in five years and 60-70% in 10 years (reviewed in Bingley *et al* (1993). As an alternative to population screening, we have studied this well-defined at risk-group of subjects who have been prospectively followed, to compare the predictive power of IAR Ab and IA-2 Ab.

Measurement of IAR Ab and IA-2 Ab in these sera has allowed us direct comparison of the abilities of these assays to predict IDDM, using identical assay formats and standardised reference ranges. In total, 33 of these subjects have developed IDDM. IAR and IA-2 antibodies were measured in 115 subjects with ICA $\geq$ 20 and/or IAA $\geq$ 100, who have been followed for 3.52 years (mean, range 0.30-7.28 years). Of this group, 18 progressed to IDDM during follow-up over 2.28 years (range 0.3-4.52 years).

**IAR and IA-2 antibody radiobinding assays**

Coupled *in vitro* transcription and translation was used to synthesise $^{35}$S-labelled protein, which was then immunoprecipitated with sera using a modification of the method of Bonifacio *et al* (1995). Complementary DNA (cDNA) encoding full length human IAR was cloned into the pBluescript vector under control of the T3 promoter; cDNA encoding amino acids 646-1015 of IAR, representing the intracellular region was inserted into the pGEM3 vector under control of the SP6 promoter and cDNA encoding full-length IA-2 was inserted into the pSP64 poly A vector under control of the SP6 promoter. Recombinant protein was synthesised using the Promega TNT reticulocyte lysate kit

(Promega, Madison, Wis., USA), in the presence of $^{35}S$ methionine, according to the manufacturers' instructions, using the appropriate RNA polymerase. Two $\mu$g DNA template was used in all *in vitro* translation and transcription reactions, which proceeded for two hours at 30°C.

The transcription/translation product (50$\mu$l) was diluted into 12ml TBST-BSA buffer (50mM Tris-HCl pH 7.2, 150mM NaCl, 1% Tween 20, 1mM L-methionine, 0.1% BSA), and 50$\mu$l of this dilute lysate was added to 5$\mu$l test serum and incubated overnight at 4°C in a 96-well plate (well volune 2ml, Beckman, Palo Alto, CA, USA). Fifty $\mu$l of protein A Sepharose suspension (50mg/ml dry weight) in TBST-BSA was added and the plate was shaken on a plate shaker for 1h at 4°C. Beads were washed x3 with 1ml TBST-BSA in the plate. Immunoprecipitates were transferred to a 96-well Unifilter plate (Packard, meriden, CT, USA), using a multichannel pipette, in 100$\mu$l TBST-BSA. Scintillation fluid (30$\mu$l Microscint 40, Packard) was added to the wells and radioactivity was counted in a TopCount Microplate Scintillation Counter (Packard). Results were expressed as arbitrary units, calculated using the formula:

Result= ((sample - negative control) / (positive control - negative control)).

The negative control consisted of pooled normal serum and the positive control was strongly IAR and IA-2 positive plasmapheresis fluid from a patient with stiff-man syndrome and diabetes. The inter assay coefficient of variation of the IAR antibody assay was 5.8% for a sample with a mean value of 57.3 units, and 12.5% for the IA-2 Ab assay, using a sample with a mean value of 96.8 units.

**ICA and IAA assays**

ICA were measured by indirect immunofluorescence on frozen sections of blood group O human pancreas (Bonifacio

*et al* (1990)) and assigned a value in JDF units by dilution and comparison with standard sera. This assay has been standardised in all Immunology of Diabetes and Proficiency Workshops. Insulin autoantibodies were measured by a competitive fluid phase radiobinding assay (Vardi *et al* (1989)).

**Intravenous glucose tolerance test (IVGTT)**

The Islet Cell Antibody Registry Users Study (ICARUS) recommended protocol was used for the IVGTT, as previously described (McNair *et al* (1995)). Serum insulin was measured using a commercial RIA kit (Pharmacia, Uppsala, Sweden).

**Statistical analysis**

Associations between parameters were assessed using regression analysis. To standardise the specificty of antibody assays for comparison of sensitivity, the threshold for positivity of each assay was adjusted to derive a specificity of 97-98%. This level of specificity was chosen because it detects a sufficiently small number of false positives to be useful in population screening. The effect of IAR and IA-2 antibody positivity on IDDM-free survival was analysed using the log-rank test.

**Results**

Before comparing IAR Ab with IA-2 Ab, the partial (intracellular domain) and full-length IAR clones were compared for recognition by IDDM sera, to determine whether additional reactivity was present on the extracellular domain of the full-length IAR molecule that was not present in the intracellular (cytoplasmic) domain. There was a strong relationship between levels of antibodies to full-length IAR, and antibodies to the intracellular domain

(r=94.9%, p<0.0001). Use of a standardised specificity of 97.9% resulted in a sensitivity of 58.1% for antibodies to the cytoplasmic domain and 48.8% for antibodies to the full-length molecule. Because of the strong relationship between antibody levels and the similar assay sensitivities, the intracellular fragment was used as the antigen in the remaining experiments.

IAR Ab and IA-2 Ab were measured in 53 recent-onset IDDM and 144 healthy control subjects. The reference range for each assay was adjusted to give a specififcity of 97.9% for each assay, which was 0.5 units for IAR Ab and 3.0 units for IA-2 Ab. The corresponding sensitivities were 56.6% for IAR Ab and 62.3% for IA-2 Ab (Table 2). Sensitivities and specificities of combinations of IAR Ab and IA-2 Ab, and IAR Ab or IA-2 Ab, using the reference ranges described above, are also shown in Table 2. The presence of either antibody resulted in a sensitivity of 73.6% and a specificity of 96.5%, and the presence of both antibodies gave a specificity of 100% and a sensitivity of 45.3%. Although levels of IAR Ab and IA-2 Ab were correlated in recent-onset subjects (r=0.7, p<0.0001, Figure 9) and there was a degree of overlap, some patients were positive for one antibody only. Thus, six (11.3%) were positive for IAR Ab and negative for IA-2 Ab, and nine (17%) were positive for IA-2 Ab and negative for IAR Ab. In some cases there were marked differences between levels of IAR Ab and IA-2 Ab, for example there were six sera with IA-2 Ab levels greater than 50 units and IAR Ab less than 5 units, and one with IAR Ab of 25 units and IA-2 Ab of 2.9 units.

We next examined the ability of IAR Ab and IA-2 to discriminate between IDDM progression and non-progression, in our prospective study of first-degree relatives with ICA $\geq$ 20JDFU or IAA $\geq$ 100nU/ml. IAR and IA-2 antibody positivity and combinations of these antibodies are summarised in Table 2. Of the 18 subjects who progressed to IDDM, IAR Ab were detectable in nine (50.0%) and IA-2 Ab

in 10 (55.6%) 2.6± 1.8 (mean, range 0.3-6.3) years prior to diagnosis, using the threshold for positivity defined above. Of the 97 nonprogressors, only 21 (21.6%) had IAR antibodies, whereas 31 (31.9%) had IA-2 antibodies. The difference in IDDM-free survival between IAR Ab positive and negative subjects was statistically significant ($p<0.001$ log-rank test), whereas IA-2 Ab positivity did not significantly influence IDDM-free survival. The combination of either IAR or IA-2 antibodies did not significantly affect IDDM-free survival, but the presence of both antibodies had a significant effect ($p<0.0005$). Although IA-2 Ab positivity did not affect IDDM-free survival when these standardised thresholds of positivity were used, the use of higher thresholds for IA-2 Ab positivity resulted in significant differences. For example, when nine units was used as the threshold, IA-2 Ab positivity was significantly associated with progression to IDDM ($p<0.0005$, log-rank test).

In addition to predicting the development of IDDM, IAR Ab were associated with markers of high risk of IDDM, in relatives with ICA or IAA. Insulin autoantibodies (Ziegler *et al* (1989)) and impaired intravenous glucose tolerance (Vardi *et al* (1991)) have both been previously shown to be associated with progression to IDDM in ICA positive first-degree relatives. IAA were significantly correlated with IAR antibodies ($r=0.25$, $p=0.005$) but not with IA-2 antibodies ($r=0.11$, N.S.). Both IAR and IA-2 antibodies were negatively correlated with the sum of the 1 and 3 minute insulin levels during an intravenous glucose tolerance test (IAR: $r=0.27$, $p<0.01$; IA-2: $r=0.25$, $p<0.05$). In addition to these relationships, both IAR and IA-2 antibodies were strongly correlated with ICA levels (IAR: $r=0.31$, $p<0.001$, IA-2:$r=0.38$).

There were some subjects in whom there were marked differences in the level of each antibody. Furthermore, in our group of high-risk ICA or IAA positive first degree relatives, the presence of IAR Ab was associated with

progression to IDDM whereas IA-2 Ab was not. Neither combinations of IAR Ab or IA-2 Ab, nor IAR Ab and IA-2 Ab resulted in better prediction of IDDM than IAR Ab alone in the prospective study; thus, combined measurement of PTP antibodies does not appear to be preferable to measurement of IAR Ab alone in IDDM screening programmes. In addition to discriminating subjects who progressed to IDDM, IAR Ab were correlated with other markers of progression to IDDM, namely IAA and impaired first-phase insulin release. These results provide evidence that IAR Ab are markers of $\beta$ cell destruction.

Levels of antibodies against the cytoplasmic domain of IAR were closely correlated with those against the full-length clone, and sensitivities of both assays were similar, suggesting that antibodies from IDDM patients recognise the cytoplasmic domain of IAR.

In conclusion, we have demonstrated that IAR Ab have similar diagnostic sensitivity for recent-onset IDDM to IA-2 Ab, but are better predictors of IDDM in our high-risk subjects. As preclinical intervention therapies in IDDM are critically reliant on effective screening tests to define high-risk subjects, IAR Ab may well have an important role in IDDM screening, either alone or in combination with other humoral markers of IDDM.

TABLE 2

| | IAR Ab | IA-2 Ab | IAR Ab or IA-2 Ab | IAR Ab & IA-2 Ab |
|---|---|---|---|---|
| Positivity threshold (units) | <0.5 | <3.0 | | |
| *Recent-onset IDDM* | | | | |
| Sensitivity (%) | 56.6 | 62.3 | 73.6 | 45.3 |
| Specificity (%) | 97.9 | 97.9 | 96.5 | 100.0 |
| ICA *positive first-degree relatives* | | | | |
| % Progressors positive | 55.6 | 50.0 | 61.1 | 44.4 |
| % Nonprogressors positive | 21.6 | 32.0 | 39.2 | 13.4 |
| *p*(logrank test) | <0.001 | N.S. | N.S | 0.0005 |

**Table 2 Comparison of diagnostic sensitivity and predictive power for IDDM, of IAR Ab, IA-2 Ab and combinations of IAR Ab and/or IA-2 Ab.**

**Thresholds for positivity described in text**

**References:**

Adams, M.D., Kerlavage, A.R., Fields, C. and Venter, J.C. (1993). Nature Genet. 4, 256-267.

Guan, K. and Dixon, J.E. (1991). Analyt. Biochem. 192, 262-267.

Lan, M.S., Lu, J., Goto, Y. and Notkins, A.L. (1994). DNA Cell Biol. 13, 505-514.

Lu, J., Notkins, A.L. and Lan, M.S. (1994). Biochem. Biophys. Res. Commun. 204, 930-936.

Rabin, D.U., Pleasic, S.M., Shapiro, J.A., Yoo-Warren, H., Oles, J., Hicks, J.M., Goldstein, D.E. and Rae, P.M.M.

(1994). J. Immunol.152, 3183-3188.
Smith, D.B. and Johnson, K.S. (1988). Gene 67, 31-40.
Walker, P.A., Leong, L. E.-C., Ng, P.W.P., Tan, S.H., Waller, S., Murphy, D. and Porter, A.G. (1994). Bio/Technology 12, 601-605.
Wang, Y. and Pallen, C.J. (1991). EMBO J. 10, 3231-3237.
Kozak, M. (1989) J. Cell Biol. 108, 229-241.
von Heijne, G. (1986) Nuc. Acids Res. 14, 4683-4690.
Kambayashi, Y., Takahashi, K., Bardhan, S., & Inagami, T. (1995) Biochem. J. 306, 331-335.
Bonifacio, E., Bingley, P.J., Shattock, M., Dean, B.M., Dunger, D., Gale, E.A.M., & Bottazzo, G.F. (1990) Lancet 335, 147-149.
Schmidli, R.S., DeAizpurua, H.J., Harrison, L.C. & Colman, P.G. (1994) J. Autoimmun. 7, 55-66.
Bingley P.J., Bonifacio E., Gale E.A.M. (1993) Diabetes 42:213-20.
Ziegler A.G., Ziegler R., Vardi P., Jackson RA, Soeldner J.S., Eisenbarth G.S.(1989). Diabetes 38:1320-5.
Bonifacio E., Genovese S., Braghi S., Bazzigaluppi E., Lampasona V., Bingley P.J. *et al*, (1995). Diabetologia 38:816-822.
Vardi P., Dib S.A., Tuttleman M., Connelly J.E., Grinbergs M, Radizabeh A., Riley W.J., Maclaren N.K., Eisenbarth G.S., Soeldner J.S., (1987). Diabetes 36:1286-91.
McNair P.D., Colman P,G, Alford FP, Harrison LC (1995). Diabetes Care 18:1168-73.
Vardi P., Crisa L., Jackson R.A. (1991) Diabetologia 34:93-102.

SEQUENCE INFORMATION

SEQ. ID. Nos. 1 and 2: partial cDNA sequence of IAR-PTP (SEQ ID NO. 1) and encoded amino acid sequence (SEQ ID NO. 2).

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AGT CGG GAG GGC GGT GCT GCC CTG GCC AAC GCC CTC CGA CGC CAC CTG
Ser Arg Glu Gly Gly Ala Ala Leu Ala Asn Ala Leu Arg Arg His Leu
                 5                  10                  15

CCC TTC CTG GAG GCC CTG TCC CAG GCC CCA GCC TCA GAC GTG CTC GCC
Pro Phe Leu Glu Ala Leu Ser Gln Ala Pro Ala Ser Asp Val Leu Ala
            20                  25                  30

AGG ACC CAT ACG GCG CAG GAC AGA CCC CCC GCT GAG GGT GAT GAC CGC
Arg Thr His Thr Ala Gln Asp Arg Pro Pro Ala Glu Gly Asp Asp Arg
        35                  40                  45

TTC TCC GAG AGC ATC CTG ACC TAT GTG GCC CAC ACG TCT GCG CTG ACC
Phe Ser Glu Ser Ile Leu Thr Tyr Val Ala His Thr Ser Ala Leu Thr
    50                  55                  60

TAC CCT CCC GGG TCC CGG ACC CAG CTC CGC GAG GAC CTC CTG CCG CGG
Tyr Pro Pro Gly Ser Arg Thr Gln Leu Arg Glu Asp Leu Leu Pro Arg
65                  70                  75                  80

ACC CTC GGC CAG CTC CAG CCA GAT GAG CTC AGC CCT AAG GTG GAC AGT
Thr Leu Gly Gln Leu Gln Pro Asp Glu Leu Ser Pro Lys Val Asp Ser
                85                  90                  95

GGT GTG GAC AGA CAC CAT CTG ATG GCG GCC CTC AGT GCC TAT GCT GCC
Gly Val Asp Arg His His Leu Met Ala Ala Leu Ser Ala Tyr Ala Ala
            100                 105                 110

CAG AGG CCC CCA GCT CCC CCC GGG GAG GGC AGC CTG GAG CCA CAG TAC
Gln Arg Pro Pro Ala Pro Pro Gly Glu Gly Ser Leu Glu Pro Gln Tyr
        115                 120                 125

CTT CTG CGT GCA CCC TCA AGA ATG CCC AGG CCT TTG CTG GCA CCA GCC
Leu Leu Arg Ala Pro Ser Arg Met Pro Arg Pro Leu Leu Ala Pro Ala
    130                 135                 140

GCC CCC CAG AAG TGG CCT TCA CCT CTG GGA GAT TCC GAA GAC CCC TCC
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Ala Pro Gln Lys Trp Pro Ser Pro Leu Gly Asp Ser Glu Asp Pro Ser
145 150 155 160

AGC ACA GGC GAT GGA GCA CGG ATT CAT ACC CTC CTG AAG GAC CTG CAG

Ser Thr Gly Asp Gly Ala Arg Ile His Thr Leu Leu Lys Asp Leu Gln
165 170 175

AGG CAG CCG GCT GAG GTG AGG GGC CTG AGT GGC CTG GAG CTG GAC GGC

Arg Gln Pro Ala Glu Val Arg Gly Leu Ser Gly Leu Glu Leu Asp Gly
180 185 190

ATG GCT GAG CTG ATG GCT GGC CTG ATG CAA GGC GTG GAC CAT GGA GTA

Met Ala Glu Leu Met Ala Gly Leu Met Gln Gly Val Asp His Gly Val
195 200 205

GCT CGA GGC AGC CCT GGG AGA GCG GCC CTG GGA GAG TCT GGA GAA CAG

Ala Arg Gly Ser Pro Gly Arg Ala Ala Leu Gly Glu Ser Gly Glu Gln
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Ala Asp Gly Pro Lys Ala Thr Leu Arg Gly Asp Ser Phe Pro Asp Asp
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Gly Val Gln Asp Asp Asp Asp Arg Leu Tyr Gln Glu Val His Arg Leu
245 250 255

AGT GCC ACA CTC GGG GGC CTC CTG CAG GAC CAC GGG TCT CGA CTC TTA

Ser Ala Thr Leu Gly Gly Leu Leu Gln Asp His Gly Ser Arg Leu Leu
260 265 270

CCT GGA GCC CTC CCC TTT GCA AGG CCC CTC GAC ATG GAG AGG AAG AAG

Pro Gly Ala Leu Pro Phe Ala Arg Pro Leu Asp Met Glu Arg Lys Lys
275 280 285

TCC GAG CAC CCT GAG TCT TCC CTG TCT TCA GAA GAG GAG ACT GCC GGA

Ser Glu His Pro Glu Ser Ser Leu Ser Ser Glu Glu Glu Thr Ala Gly
290 295 300

GTG GAG AAC GTC AAG AGC CAG ACG TAT TCC AAA GAT CTG CTG GGG CAG

Val Glu Asn Val Lys Ser Gln Thr Tyr Ser Lys Asp Leu Leu Gly Gln
305 310 315 320

CAG CCG CAT TCG GAG CCC GGG GCC GCT GCG TTT GGG GAG CTC CAA AAC

Gln Pro His Ser Glu Pro Gly Ala Ala Ala Phe Gly Glu Leu Gln Asn

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        325                         330                         335

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        340                 345                 350

CAG GAG GCC CTC AGC GAC GGC CTG CAA TTG GAG GTC CAG CCT TCC GAG

Gln Glu Ala Leu Ser Asp Gly Leu Gln Leu Glu Val Gln Pro Ser Glu
    355                 360                 365

GAA GAG GCG CGG GGC TAC ATC GTG ACA GAC AGA GAC CCC CTG CGC CCC

Glu Glu Ala Arg Gly Tyr Ile Val Thr Asp Arg Asp Pro Leu Arg Pro
370                 375                 380

GAG GAA GGA AGG CGG CTG GTG GAG GAC GTC GCC CGC CTC CTG CAG GTG

Glu Glu Gly Arg Arg Leu Val Glu Asp Val Ala Arg Leu Leu Gln Val
385                 390                 395                 400

CCC AGC AGC GCG TTC GCT GAC GTG GAG GTT CTC GGA CCA GCA GTG ACC

Pro Ser Ser Ala Phe Ala Asp Val Glu Val Leu Gly Pro Ala Val Thr
            405                 410                 415

TTC AAA GTG AGC GCC AAT GTC CAA AAC GTG ACC ACT GAG GAT GTG GAG

Phe Lys Val Ser Ala Asn Val Gln Asn Val Thr Thr Glu Asp Val Glu
        420                 425                 430

AAG GCC ACA GTT GAC AAC AAA GAC AAA CTG GAG GAA ACC TCT GGA CTG

Lys Ala Thr Val Asp Asn Lys Asp Lys Leu Glu Glu Thr Ser Gly Leu
    435                 440                 445

AAA ATT CTT CAA ACC GGA GTC GGG TCG AAA AGC AAA CTC AAG TTC CTG

Lys Ile Leu Gln Thr Gly Val Gly Ser Lys Ser Lys Leu Lys Phe Leu
450                 455                 460

CCT CCT CAG GCG GAG CAA GAA GAC TCC ACC AAG TTC ATC GCG CTC ACC

Pro Pro Gln Ala Glu Gln Glu Asp Ser Thr Lys Phe Ile Ala Leu Thr
465                 470                 475                 480

CTG GTC TCC CTC GCC TGC ATC CTG GGC GTC CTC CTG GCC TCT GGC CTC

Leu Val Ser Leu Ala Cys Ile Leu Gly Val Leu Leu Ala Ser Gly Leu
            485                 490                 495

ATC TAC TGC CTC CGC CAT AGC TCT CAG CAC AGG CTG AAG GAG AAG CTC

Ile Tyr Cys Leu Arg His Ser Ser Gln His Arg Leu Lys Glu Lys Leu
        500                 505                 510
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Ser Gly Leu Gly Gly Asp Pro Gly Ala Asp Ala Thr Ala Ala Tyr Gln
    515                 520                 525

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Glu Leu Cys Arg Gln Arg Met Ala Thr Arg Pro Pro Asp Arg Pro Glu
530                 535                 540

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Gly Pro His Thr Ser Arg Ile Ser Ser Val Ser Ser Gln Phe Ser Asp
545                 550                 555                 560

GGG CCG ATC CCC AGC CCC TCC GCA CGC AGC AGC GCC TCA TCC TGG TCC
Gly Pro Ile Pro Ser Pro Ser Ala Arg Ser Ser Ala Ser Ser Trp Ser
            565                 570                 575

GAG GAG CCT GTG CAG TCC AAC ATG GAC ATC TCC ACC GGC CAC ATG ATC
Glu Glu Pro Val Gln Ser Asn Met Asp Ile Ser Thr Gly His Met Ile
        580                 585                 590

CTG TCC TAC ATG GAG GAC CAC CTG AAG AAC AAG AAC CGG CTG GAG AAG
Leu Ser Tyr Met Glu Asp His Leu Lys Asn Lys Asn Arg Leu Glu Lys
    595                 600                 605

GAG TGG GAA GCG CTG TGC GCC TAC CAG GCG GAG CCC AAC AGC TCG TTC
Glu Trp Glu Ala Leu Cys Ala Tyr Gln Ala Glu Pro Asn Ser Ser Phe
610                 615                 620

GTG GCC CAG AGG GAG GAG AAC GTG CCC AAG AAC CGC TCC CTG GCC GTG
Val Ala Gln Arg Glu Glu Asn Val Pro Lys Asn Arg Ser Leu Ala Val
625                 630                 635                 640

CTG ACC TAT GAC CAC TCC CGG GTC CTG CTG AAG GCG GAG AAC AGC CAC
Leu Thr Tyr Asp His Ser Arg Val Leu Leu Lys Ala Glu Asn Ser His
            645                 650                 655

AGC CAC TCA GAC TAC ATC AAC GCT AGC CCC ATC ATG GAT CAC GAC CCG
Ser His Ser Asp Tyr Ile Asn Ala Ser Pro Ile Met Asp His Asp Pro
        660                 665                 670

AGG AAC CCC GCG TAC ATC GCC ACC CAG GGA CCG CTG CCC GCC ACC GTG
Arg Asn Pro Ala Tyr Ile Ala Thr Gln Gly Pro Leu Pro Ala Thr Val
    675                 680                 685
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GCT GAC TTT TGG CAG ATG GTG TGG GAG AGC GGC TGC GTG GTG ATC GTC

Ala Asp Phe Trp Gln Met Val Trp Glu Ser Gly Cys Val Val Ile Val
690 695 700

ATG CTG ACA CCC CTC GCG GAG AAC GGC GTC CGG CAG TGC TAC CAC TAC

Met Leu Thr Pro Leu Ala Glu Asn Gly Val Arg Gln Cys Tyr His Tyr
705 710 715 720

TGG CCG GAT GAA GGC TCC AAT CTC TAC CAC ATC TAT GAG GTG AAC CTG

Trp Pro Asp Glu Gly Ser Asn Leu Tyr His Ile Tyr Glu Val Asn Leu
725 730 735

GTC TCC GAG CAC ATC TGG TGT GAG GAC TTC CTG GTG AGG AGC TTC TAT

Val Ser Glu His Ile Trp Cys Glu Asp Phe Leu Val Arg Ser Phe Tyr
740 745 750

CTG AAG AAC CTG CAG ACC AAC GAG ACG CGC ACC GTG ACG CAG TTC CAC

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755 760 765

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Phe Leu Ser Trp Tyr Asp Arg Gly Val Pro Ser Ser Ser Arg Ser Leu
770 775 780

CTG GAC TTC CGC AGA AAA GTA AAC AAG TGC TAC AGG GGC CGT TCT TGT

Leu Asp Phe Arg Arg Lys Val Asn Lys Cys Tyr Arg Gly Arg Ser Cys
785 790 795 800

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Pro Ile Ile Val His Cys Ser Asp Gly Ala Gly Arg Ser Gly Thr Tyr
805 810 815

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Val Leu Ile Asp Met Val Leu Asn Lys Met Ala Lys Gly Ala Lys Glu
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Ile Asp Ile Ala Ala Thr Leu Glu His Leu Arg Asp Gln Arg Pro Gly
835 840 845

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850 855 860

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Glu Glu Val Asn Ala Ile Leu Lys Ala Leu Pro Gln
865 870 875

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TTAATTGTGT GTCTTCTATT ATAACTGCAT AGTAATAGGG CCCTTAGCTC TCCCGTAGTC

AGCGCAGTTT AGCAGTTAAA AGTGTATTTT TGTTTAATCA AACAATAATA AAGAGAGGTT

TGTCGGAAAA ATCCAAAAAA AA

**SEQ. ID. Nos. 10 and 11 Complete nucleotide (SEQ ID No. 10) and amino acid (SEQ ID NO. 11) sequence of IAR.**

In SEQ ID No. 11, the signal peptide is underlined and the transmembrane region is double-underlined. An extracellular RGDS sequence and a potential site of N-linked glycosylation are in bold. The active site of the phosphatase is found at amino acids 942-954.

GCCTCCCGCC GCCTCCCGCG CGGCCATGGA CTGAGCGCCG CCGGCCAGGC CGCGGGG

ATG GGG CCG CCG CTC CCG CTG CTG CTG CTG CTA CTG CTG CTG CTG CCG
105

<u>Met Gly Pro Pro Leu Pro Leu Leu Leu Leu Leu Leu Leu Leu Leu Pro</u>
1 5 10 15

CCA CGC GTC CTG CCT GCC GCC CCT TCG TCC GTC CCC CGC GGC CGG CAG

<u>Pro Arg Val Leu Pro</u> Ala Ala Pro Ser Ser Val Pro Arg Gly Arg Gln
20 25 30

CTC CCG GGG CGT CTG GGC TGC CTG CTC GAG GAG GGC CTC TGC GGA GCG

Leu Pro Gly Arg Leu Gly Cys Leu Leu Glu Glu Gly Leu Cys Gly Ala
35 40 45

TCC GAG GCC TGT GTG AAC GAT GGA GTG TTT GGA AGG TGC CAG AAG GTT

Ser Glu Ala Cys Val Asn Asp Gly Val Phe Gly Arg Cys Gln Lys Val
50 55 60

CCG GCA ATG GAC TTT TAC CGC TAC GAG GTG TCG CCC GTG GCC CTG CAG

Pro Ala Met Asp Phe Tyr Arg Tyr Glu Val Ser Pro Val Ala Leu Gln
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CGC CTG CGC GTG GCG TTG CAG AAG CTT TCC GGC ACA GGT TTC ACG TGG
Arg Leu Arg Val Ala Leu Gln Lys Leu Ser Gly Thr Gly Phe Thr Trp
85 90 95

CAG GAT GAC TAT ACT CAG TAT GTG ATG GAC CAG GAA CTT GCA GAC CTC
Gln Asp Asp Tyr Thr Gln Tyr Val Met Asp Gln Glu Leu Ala Asp Leu
100 105 110

CCG AAA ACC TAC CTG AGG CGT CCT GAA GCA TCC AGC CCA GCC AGG CCC
Pro Lys Thr Tyr Leu Arg Arg Pro Glu Ala Ser Ser Pro Ala Arg Pro
115 120 125

TCA AAA CAC AGC GTT GGC AGC GAG AGG AGG TAC AGT CGG GAG GGC GGT
Ser Lys His Ser Val Gly Ser Glu Arg Arg Tyr Ser Arg Glu Gly Gly
130 135 140

GCT GCC CTG GCC AAC GCC CTC CGA CGC CAC CTG CCC TTC CTG GAG GCC
Ala Ala Leu Ala Asn Ala Leu Arg Arg His Leu Pro Phe Leu Glu Ala
145 150 155 160

CTG TCC CAG GCC CCA GCC TCA GAC GTG CTC GCC AGG ACC CAT ACG GCG
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165 170 175

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Gln Asp Arg Pro Pro Ala Glu Gly Asp Asp Arg Phe Ser Glu Ser Ile
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210 215 220

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Gln Pro Asp Glu Leu Ser Pro Lys Val Asp Ser Gly Val Asp Arg His
225 230 235 240

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His Leu Met Ala Ala Leu Ser Ala Tyr Ala Ala Gln Arg Pro Pro Ala
245 250 255

CCC CCC GGG GAG GGC AGC CTG GAG CCA CAG TAC CTT CTG CGT GCA CCC

Pro Pro Gly Glu Gly Ser Leu Glu Pro Gln Tyr Leu Leu Arg Ala Pro
260 265 270

TCA AGA ATG CCC AGG CCT TTG CTG GCA CCA GCC GCC CCC CAG AAG TGG

Ser Arg Met Pro Arg Pro Leu Leu Ala Pro Ala Ala Pro Gln Lys Trp
275 280 285

CCT TCA CCT CTG GGA GAT TCC GAA GAC CCC TCC AGC ACA GGC GAT GGA

Pro Ser Pro Leu Gly Asp Ser Glu Asp Pro Ser Ser Thr Gly Asp Gly
290 295 300

GCA CGG ATT CAT ACC CTC CTG AAG GAC CTG CAG AGG CAG CCG GCT GAG

Ala Arg Ile His Thr Leu Leu Lys Asp Leu Gln Arg Gln Pro Ala Glu
305 310 315 320

GTG AGG GGC CTG AGT GGC CTG GAG CTG GAC GGC ATG GCT GAG CTG ATG

Val Arg Gly Leu Ser Gly Leu Glu Leu Asp Gly Met Ala Glu Leu Met
325 330 335

GCT GGC CTG ATG CAA GGC GTG GAC CAT GGA GTA GCT CGA GGC AGC CCT

Ala Gly Leu Met Gln Gly Val Asp His Gly Val Ala Arg Gly Ser Pro
340 345 350

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Gly Arg Ala Ala Leu Gly Glu Ser Gly Glu Gln Ala Asp Gly Pro Lys
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Ala Thr Leu Arg Gly Asp Ser Phe Pro Asp Asp Gly Val Gln Asp Asp
370 375 380

GAT GAT AGA CTT TAC CAA GAG GTC CAT CGT CTG AGT GCC ACA CTC GGG

Asp Asp Arg Leu Tyr Gln Glu Val His Arg Leu Ser Ala Thr Leu Gly
385 390 395 400

GGC CTC CTG CAG GAC CAC GGG TCT CGA CTC TTA CCT GGA GCC CTC CCC

Gly Leu Leu Gln Asp His Gly Ser Arg Leu Leu Pro Gly Ala Leu Pro
405 410 415

TTT GCA AGG CCC CTC GAC ATG GAG AGG AAG AAG TCC GAG CAC CCT GAG

Phe Ala Arg Pro Leu Asp Met Glu Arg Lys Lys Ser Glu His Pro Glu
420 425 430

TCT TCC CTG TCT TCA GAA GAG GAG ACT GCC GGA GTG GAG AAC GTC AAG

Ser Ser Leu Ser Ser Glu Glu Glu Thr Ala Gly Val Glu Asn Val Lys
435 440 445

AGC CAG ACG TAT TCC AAA GAT CTG CTG GGG CAG CAG CCG CAT TCG GAG

Ser Gln Thr Tyr Ser Lys Asp Leu Leu Gly Gln Gln Pro His Ser Glu
450 455 460

CCC GGG GCC GCT GCG TTT GGG GAG CTC CAA AAC CAG ATG CCT GGG CCC

Pro Gly Ala Ala Ala Phe Gly Glu Leu Gln Asn Gln Met Pro Gly Pro
465 470 475 480

TCG AAG GAG GAG CAG AGC CTT CCA GCG GGT GCT CAG GAG GCC CTC AGC

Ser Lys Glu Glu Gln Ser Leu Pro Ala Gly Ala Gln Glu Ala Leu Ser
485 490 495

GAC GGC CTG CAA TTG GAG GTC CAG CCT TCC GAG GAA GAG GCG CGG GGC

Asp Gly Leu Gln Leu Glu Val Gln Pro Ser Glu Glu Glu Ala Arg Gly
500 505 510

TAC ATC GTG ACA GAC AGA GAC CCC CTG CGC CCC GAG GAA GGA AGG CGG

Tyr Ile Val Thr Asp Arg Asp Pro Leu Arg Pro Glu Glu Gly Arg Arg
515 520 525

CTG GTG GAG GAC GTC GCC CGC CTC CTG CAG GTG CCC AGC AGC GCG TTC

Leu Val Glu Asp Val Ala Arg Leu Leu Gln Val Pro Ser Ser Ala Phe
530 535 540

GCT GAC GTG GAG GTT CTC GGA CCA GCA GTG ACC TTC AAA GTG AGC GCC

Ala Asp Val Glu Val Leu Gly Pro Ala Val Thr Phe Lys Val Ser Ala
545 550 555 560

AAT GTC CAA **AAC GTG ACC** ACT GAG GAT GTG GAG AAG GCC ACA GTT GAC

Asn Val Gln **Asn Val Thr** Thr Glu Asp Val Glu Lys Ala Thr Val Asp
565 570 575

AAC AAA GAC AAA CTG GAG GAA ACC TCT GGA CTG AAA ATT CTT CAA ACC

Asn Lys Asp Lys Leu Glu Glu Thr Ser Gly Leu Lys Ile Leu Gln Thr
580 585 590

GGA GTC GGG TCG AAA AGC AAA CTC AAG TTC CTG CCT CCT CAG GCG GAG

Gly Val Gly Ser Lys Ser Lys Leu Lys Phe Leu Pro Pro Gln Ala Glu
595 600 605

CAA GAA GAC TCC ACC AAG TTC ATC GCG CTC ACC CTG GTC TCC CTC GCC

Gln Glu Asp Ser Thr Lys Phe Ile Ala Leu Thr Leu Val Ser Leu Ala
610 615 620

TGC ATC CTG GGC GTC CTC CTG GCC TCT GGC CTC ATC TAC TGC CTC CGC

Cys Ile Leu Gly Val Leu Leu Ala Ser Gly Leu Ile Tyr Cys Leu Arg
625 630 635 640

CAT AGC TCT CAG CAC AGG CTG AAG GAG AAG CTC TCG GGA CTA GGG GGC

His Ser Ser Gln His Arg Leu Lys Glu Lys Leu Ser Gly Leu Gly Gly
645 650 655

GAC CCA GGT GCA GAT GCC ACT GCC GCC TAC CAG GAG CTG TGC CGC CAG

Asp Pro Gly Ala Asp Ala Thr Ala Ala Tyr Gln Glu Leu Cys Arg Gln
660 665 670

CGT ATG GCC ACG CGG CCA CCA GAC CGA CCT GAG GGC CCG CAC ACG TCA

Arg Met Ala Thr Arg Pro Pro Asp Arg Pro Glu Gly Pro His Thr Ser
675 680 685

CGC ATC AGC AGC GTC TCA TCC CAG TTC AGC GAC GGG CCG ATC CCC AGC

Arg Ile Ser Ser Val Ser Ser Gln Phe Ser Asp Gly Pro Ile Pro Ser
690 695 700

CCC TCC GCA CGC AGC AGC GCC TCA TCC TGG TCC GAG GAG CCT GTG CAG

Pro Ser Ala Arg Ser Ser Ala Ser Ser Trp Ser Glu Glu Pro Val Gln
705 710 715 720

TCC AAC ATG GAC ATC TCC ACC GGC CAC ATG ATC CTG TCC TAC ATG GAG

Ser Asn Met Asp Ile Ser Thr Gly His Met Ile Leu Ser Tyr Met Glu
725 730 735

GAC CAC CTG AAG AAC AAG AAC CGG CTG GAG AAG GAG TGG GAA GCG CTG

Asp His Leu Lys Asn Lys Asn Arg Leu Glu Lys Glu Trp Glu Ala Leu
740 745 750

TGC GCC TAC CAG GCG GAG CCC AAC AGC TCG TTC GTG GCC CAG AGG GAG

Cys Ala Tyr Gln Ala Glu Pro Asn Ser Ser Phe Val Ala Gln Arg Glu
755 760 765

GAG AAC GTG CCC AAG AAC CGC TCC CTG GCC GTG CTG ACC TAT GAC CAC

Glu Asn Val Pro Lys Asn Arg Ser Leu Ala Val Leu Thr Tyr Asp His
770 775 780

TCC CGG GTC CTG CTG AAG GCG GAG AAC AGC CAC AGC CAC TCA GAC TAC

Ser Arg Val Leu Leu Lys Ala Glu Asn Ser His Ser His Ser Asp Tyr

785 790 795 800

ATC AAC GCT AGC CCC ATC ATG GAT CAC GAC CCG AGG AAC CC[illegible] GCG TAC

Ile Asn Ala Ser Pro Ile Met Asp His Asp Pro Arg Asn Pro Ala Tyr
805 810 815

ATC GCC ACC CAG GGA CCG CTG CCC GCC ACC GTG GCT GAC TTT TGG CAG

Ile Ala Thr Gln Gly Pro Leu Pro Ala Thr Val Ala Asp Phe Trp Gln
820 825 830

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Met Val Trp Glu Ser Gly Cys Val Val Ile Val Met Leu Thr Pro Leu
835 840 845

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Ala Glu Asn Gly Val Arg Gln Cys Tyr His Tyr Trp Pro Asp Glu Gly
850 855 860

TCC AAT CTC TAC CAC ATC TAT GAG GTG AAC CTG GTC TCC GAG CAC ATC

Ser Asn Leu Tyr His Ile Tyr Glu Val Asn Leu Val Ser Glu His Ile
865 870 875 880

TGG TGT GAG GAC TTC CTG GTG AGG AGC TTC TAT CTG AAG AAC CTG CAG

Trp Cys Glu Asp Phe Leu Val Arg Ser Phe Tyr Leu Lys Asn Leu Gln
885 890 895

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Thr Asn Glu Thr Arg Thr Val Thr Gln Phe His Phe Leu Ser Trp Tyr
900 905 910

GAC CGA GGA GTC CCT TCC TCC TCA AGG TCC CTC CTG GAC TTC CGC AGA

Asp Arg Gly Val Pro Ser Ser Ser Arg Ser Leu Leu Asp Phe Arg Arg
915 920 925

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Lys Val Asn Lys Cys Tyr Arg Gly Arg Ser Cys Pro Ile Ile Val His
930 935 940

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Cys Ser Asp Gly Ala Gly Arg Ser Gly Thr Tyr Val Leu Ile Asp Met
945 950 955 960

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Val Leu Asn Lys Met Ala Lys Gly Ala Lys Glu Ile Asp Ile Ala Ala
965 970 975

ACC CTG GAG CAC TTG AGG GAC CAG AGA CCC GGC ATG GTC CAG ACG AAG
Thr Leu Glu His Leu Arg Asp Gln Arg Pro Gly Met Val Gln Thr Lys
980 985 990

GAG CAG TTT GAG TTC GCG CTG ACA GCC GTG GCT GAG GAG GTG AAC GCC
Glu Gln Phe Glu Phe Ala Leu Thr Ala Val Ala Glu Glu Val Asn Ala
995 1000 1005

ATC CTC AAG GCC CTT CCC CAG T GAGCGGCAGC CTCAGGGGCC TCAGGGGAGC
Ile Leu Lys Ala Leu Pro Gln
1010 1015

CCCTACCCCA CGGATGTTGT CAGGAATCAT GATCTGACTT TAATTGTGTG TCTTCTATTA

TAACTGCATA GTAATAGGGC CCTTAGCTCT CCCGTAGTCA GCGCAGTTTA GCAGTTAAAA

GTGTATTTTT GTTTAATCAA ACAATAATAA AGAGAGGTTT GTCGGAAAAA TCCAAAA

SEQ ID Nos 12 and 13: Nucleotide (SEQ ID No. 12) and amino acid (SEQ ID NO. 13) sequences of the cytoplasmic (intracellular) domain of IAR. SEQ ID NO. 12 corresponds to the nucleotides of SEQ ID No. 10 which encode amino acids 646 to 1015 of SEQ ID No. 11. SEQ ID No. 13 corresponds to amino acids 646 to 1015 of SEQ ID No. 11.

AGG CTG AAG GAG AAG CTC TCG GGA CTA GGG GGC
Arg Leu Lys Glu Lys Leu Ser Gly Leu Gly Gly
650 655

GAC CCA GGT GCA GAT GCC ACT GCC GCC TAC CAG GAG CTG TGC CGC CAG
Asp Pro Gly Ala Asp Ala Thr Ala Ala Tyr Gln Glu Leu Cys Arg Gln
660 665 670

CGT ATG GCC ACG CGG CCA CCA GAC CGA CCT GAG GGC CCG CAC ACG TCA
Arg Met Ala Thr Arg Pro Pro Asp Arg Pro Glu Gly Pro His Thr Ser
675 680 685

CGC ATC AGC AGC GTC TCA TCC CAG TTC AGC GAC GGG CCG ATC CCC AGC

Arg Ile Ser Ser Val Ser Ser Gln Phe Ser Asp Gly Pro Ile Pro Ser
690 695 700

CCC TCC GCA CGC AGC AGC GCC TCA TCC TGG TCC GAG GAG CCT GTG CAG

Pro Ser Ala Arg Ser Ser Ala Ser Ser Trp Ser Glu Glu Pro Val Gln
705 710 715 720

TCC AAC ATG GAC ATC TCC ACC GGC CAC ATG ATC CTG TCC TAC ATG GAG

Ser Asn Met Asp Ile Ser Thr Gly His Met Ile Leu Ser Tyr Met Glu
725 730 735

GAC CAC CTG AAG AAC AAG AAC CGG CTG GAG AAG GAG TGG GAA GCG CTG

Asp His Leu Lys Asn Lys Asn Arg Leu Glu Lys Glu Trp Glu Ala Leu
740 745 750

TGC GCC TAC CAG GCG GAG CCC AAC AGC TCG TTC GTG GCC CAG AGG GAG

Cys Ala Tyr Gln Ala Glu Pro Asn Ser Ser Phe Val Ala Gln Arg Glu
755 760 765

GAG AAC GTG CCC AAG AAC CGC TCC CTG GCC GTG CTG ACC TAT GAC CAC

Glu Asn Val Pro Lys Asn Arg Ser Leu Ala Val Leu Thr Tyr Asp His
770 775 780

TCC CGG GTC CTG CTG AAG GCG GAG AAC AGC CAC AGC CAC TCA GAC TAC

Ser Arg Val Leu Leu Lys Ala Glu Asn Ser His Ser His Ser Asp Tyr
785 790 795 800

ATC AAC GCT AGC CCC ATC ATG GAT CAC GAC CCG AGG AAC CCC GCG TAC

Ile Asn Ala Ser Pro Ile Met Asp His Asp Pro Arg Asn Pro Ala Tyr
805 810 815

ATC GCC ACC CAG GGA CCG CTG CCC GCC ACC GTG GCT GAC TTT TGG CAG

Ile Ala Thr Gln Gly Pro Leu Pro Ala Thr Val Ala Asp Phe Trp Gln
820 825 830

ATG GTG TGG GAG AGC GGC TGC GTG GTG ATC GTC ATG CTG ACA CCC CTC

Met Val Trp Glu Ser Gly Cys Val Val Ile Val Met Leu Thr Pro Leu
835 840 845

GCG GAG AAC GGC GTC CGG CAG TGC TAC CAC TAC TGG CCG GAT GAA GGC

Ala Glu Asn Gly Val Arg Gln Cys Tyr His Tyr Trp Pro Asp Glu Gly
850 855 860

TCC AAT CTC TAC CAC ATC TAT GAG GTG AAC CTG GTC TCC GAG CAC ATC

Ser Asn Leu Tyr His Ile Tyr Glu Val Asn Leu Val Ser Glu His Ile
865 870 875 880

TGG TGT GAG GAC TTC CTG GTG AGG AGC TTC TAT CTG AAG AAC CTG CAG

Trp Cys Glu Asp Phe Leu Val Arg Ser Phe Tyr Leu Lys Asn Leu Gln
885 890 895

ACC AAC GAG ACG CGC ACC GTG ACG CAG TTC CAC TTC CTG AGT TGG TAT

Thr Asn Glu Thr Arg Thr Val Thr Gln Phe His Phe Leu Ser Trp Tyr
900 905 910

GAC CGA GGA GTC CCT TCC TCC TCA AGG TCC CTC CTG GAC TTC CGC AGA

Asp Arg Gly Val Pro Ser Ser Ser Arg Ser Leu Leu Asp Phe Arg Arg
915 920 925

AAA GTA AAC AAG TGC TAC AGG GGC CGT TCT TGT CCA ATA ATT GTT CAT

Lys Val Asn Lys Cys Tyr Arg Gly Arg Ser Cys Pro Ile Ile Val His
930 935 940

TGC AGT GAC GGT GCA GGC CGG AGC GGC ACC TAC GTC CTG ATC GAC ATG

Cys Ser Asp Gly Ala Gly Arg Ser Gly Thr Tyr Val Leu Ile Asp Met
945 950 955 960

GTT CTC AAC AAG ATG GCC AAA GGT GCT AAA GAG ATT GAT ATC GCA GCG

Val Leu Asn Lys Met Ala Lys Gly Ala Lys Glu Ile Asp Ile Ala Ala
965 970 975

ACC CTG GAG CAC TTG AGG GAC CAG AGA CCC GGC ATG GTC CAG ACG AAG

Thr Leu Glu His Leu Arg Asp Gln Arg Pro Gly Met Val Gln Thr Lys
980 985 990

GAG CAG TTT GAG TTC GCG CTG ACA GCC GTG GCT GAG GAG GTG AAC GCC

Glu Gln Phe Glu Phe Ala Leu Thr Ala Val Ala Glu Glu Val Asn Ala
995 1000 1005

ATC CTC AAG GCC CTT CCC CAG

Ile Leu Lys Ala Leu Pro Gln
1010 1015

## CLAIMS

1. A nucleic acid encoding a polypeptide that has the properties of an Insulin-dependent diabetes mellitus (IDDM)-associated autoantigen, which nucleic acid comprises:

(a) the coding sequence of SEQ. ID. No. 1, 10 or 12, and/or a sequence complementary thereto;

(b) a sequence which hybridises to a sequence defined in (a);

(c) a sequence that is degenerate as a result of the genetic code to a nucleic acid sequence defined in (a) or (b);

(d) a sequence having at least 80% homology to a sequence defined in (a), (b) or (c).

2. A nucleic acid according to claim 1 comprising the coding sequence of SEQ. ID. No. 1, 10 or 12.

3. A polypeptide encoded by a nucleic acid according to claim 1 or 2.

4. A polypeptide according to claim 3 which comprises the amino acid sequence of SEQ. ID. No. 2, 11 or 13; or one or more parts thereof.

5. A nucleic acid vector including a nucleic acid as defined in claim 1 or 2.

6. A cell harbouring a vector according to claim 5 or a nucleic acid according to claim 1 or 2.

7. A method of producing a polypeptide according to claim 3 or 4 which comprises culturing a cell according to claim 6 under conditions which permit the expression of the polypeptide; and recovering the polypeptide.

8. A method of diagnosing IDDM which comprises:

(a) contacting a sample from an individual with a polypeptide according to claim 3 or 4 under conditions that permit the recognition of the polypeptide by autoantibodies that recognise IAR; and

(b) determining whether or not said recognition occurs.

9. A method according to claim 8 which further comprises:

(c) contacting a sample from the individual with at least one further IDDM-associated autoantigen, or a polypeptide that functions as an IDDM-associated antigen, under conditions which permit recognition of the autoantigen or polypeptide by autoantibodies; and

(d) determining whether or not said recognition occurs.

10. A method according to claim 9 wherein a further autoantigen or polypeptide is hIA-2, GAD, ICA-512, ICA-12, ICA-13, ICA-208, ICA-302, ICA-313, ICA-525 or ICA-505; or a polypeptide that functions as one of these further auto-autoantigens.

11. A method according to claim 8, 9 or 10 wherein the sample is taken from a patient who is a first-degree relation of a known IDDM sufferer.

12. Use of a polypeptide according to claim 3 or 4 in diagnosing IDDM.

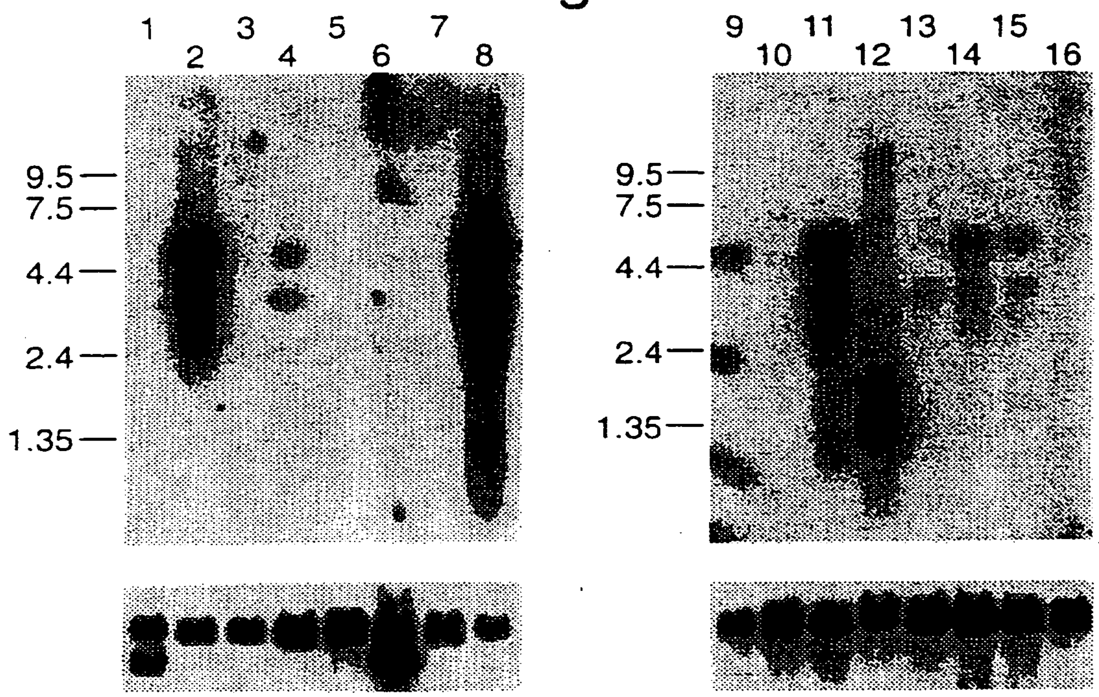
13. A diagnostic test kit for diagnosing IDDM which comprises a polypeptide according to claim 3 or 4, and means for determining whether or not the polypeptide is recognised by autoantibodies that recognise IAR.

14. A kit according to claim 13 which further comprises at least one further IDDM-associated autoantigen.

15. A kit according to claim 14 which further comprises means for determining whether or not the further autoantigen is recognised by autoantibodies.

16. A kit according to claim 14 or 15 wherein a further autoantigen or polypeptide is hIA-2, GAD, ICA-512, ICA-12, ICA-13, ICA-208, ICA-302, ICA-313, ICA-525 or ICA-505; or a polypeptide that functions as one of these further auto-autoantigens.

# Fig.1.




1. HEART
2. BRAIN
3. PLACENTA
4. LUNG
5. LIVER
6. SKELETAL MUSCLE
7. KIDNEY
8. PANCREAS

9. SPLEEN
10. THYMUS
11. PROSTATE
12. TESTIS
13. OVARY
14. SMALL INTESTINE
15. COLON
16. PERIPHERAL BLOOD LEUKOCYTE

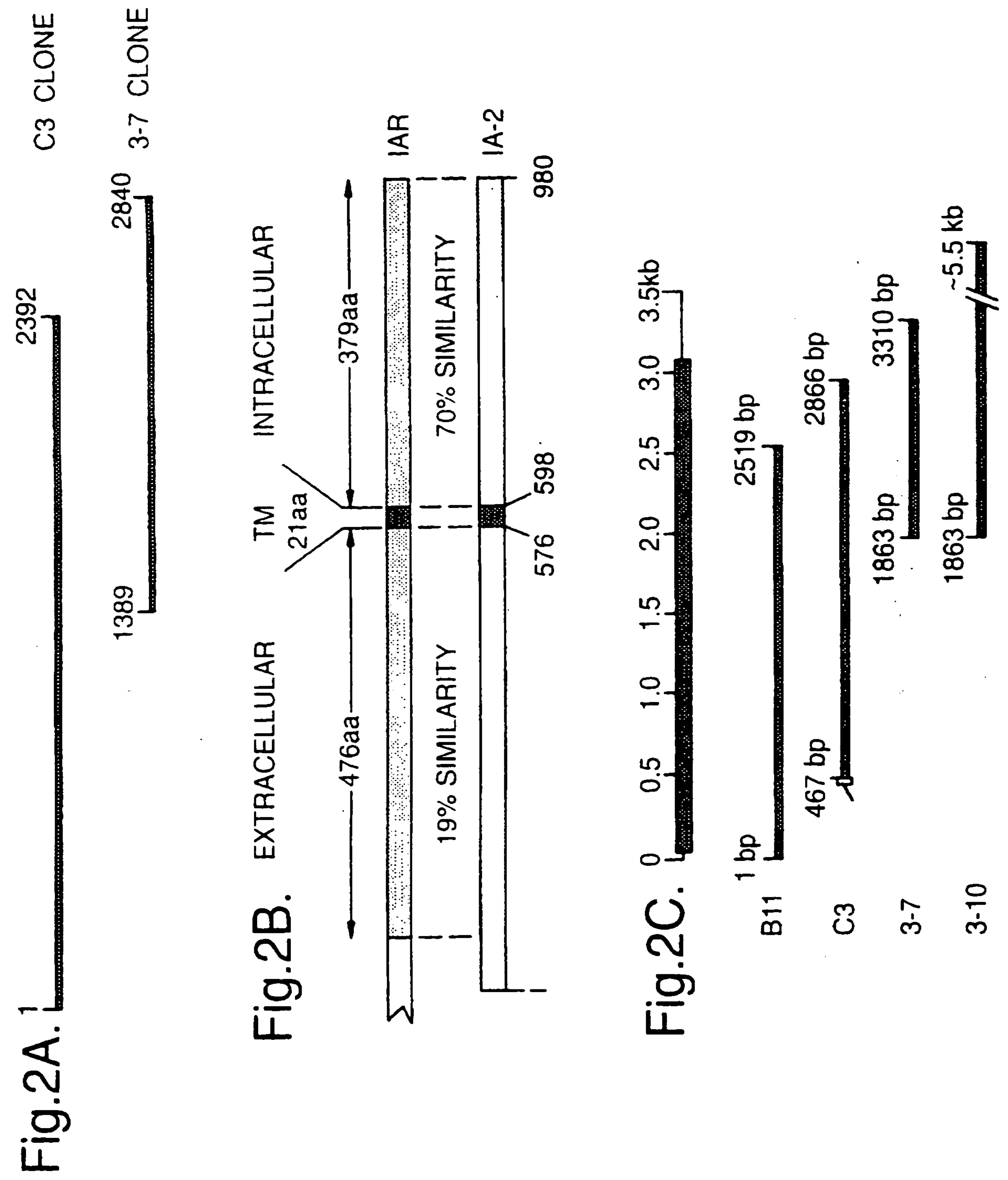
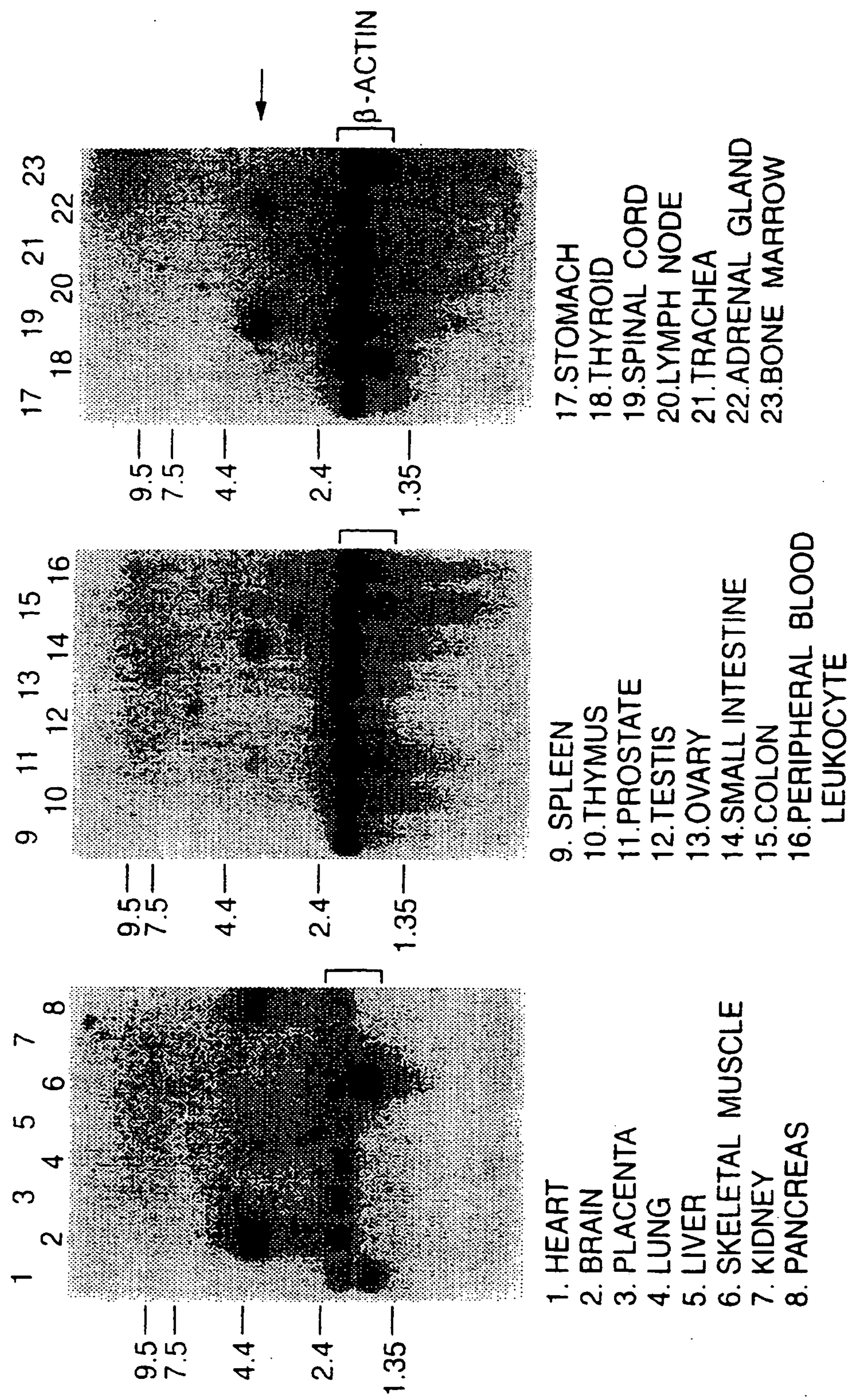



Fig.2A.

Fig.2B.

Fig.2C.

Fig.3.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9.5
7.5
4.4
2.4
1.35
1. HEART
2. BRAIN
3. PLACENTA
4. LUNG
5. LIVER
6. SKELETAL MUSCLE
7. KIDNEY
8. PANCREAS
9 10 11 12 13 14 15 16
9.5
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2.4
1.35
9. SPLEEN
10.THYMUS
11.PROSTATE
12.TESTIS
13.OVARY
14.SMALL INTESTINE
15.COLON
16.PERIPHERAL BLOOD LEUKOCYTE
17 18 19 20 21 22 23
β-ACTIN
9.5
7.5
4.4
2.4
1.35
17.STOMACH
18.THYROID
19.SPINAL CORD
20.LYMPH NODE
21.TRACHEA
22.ADRENAL GLAND
23.BONE MARROW

Fig.4.

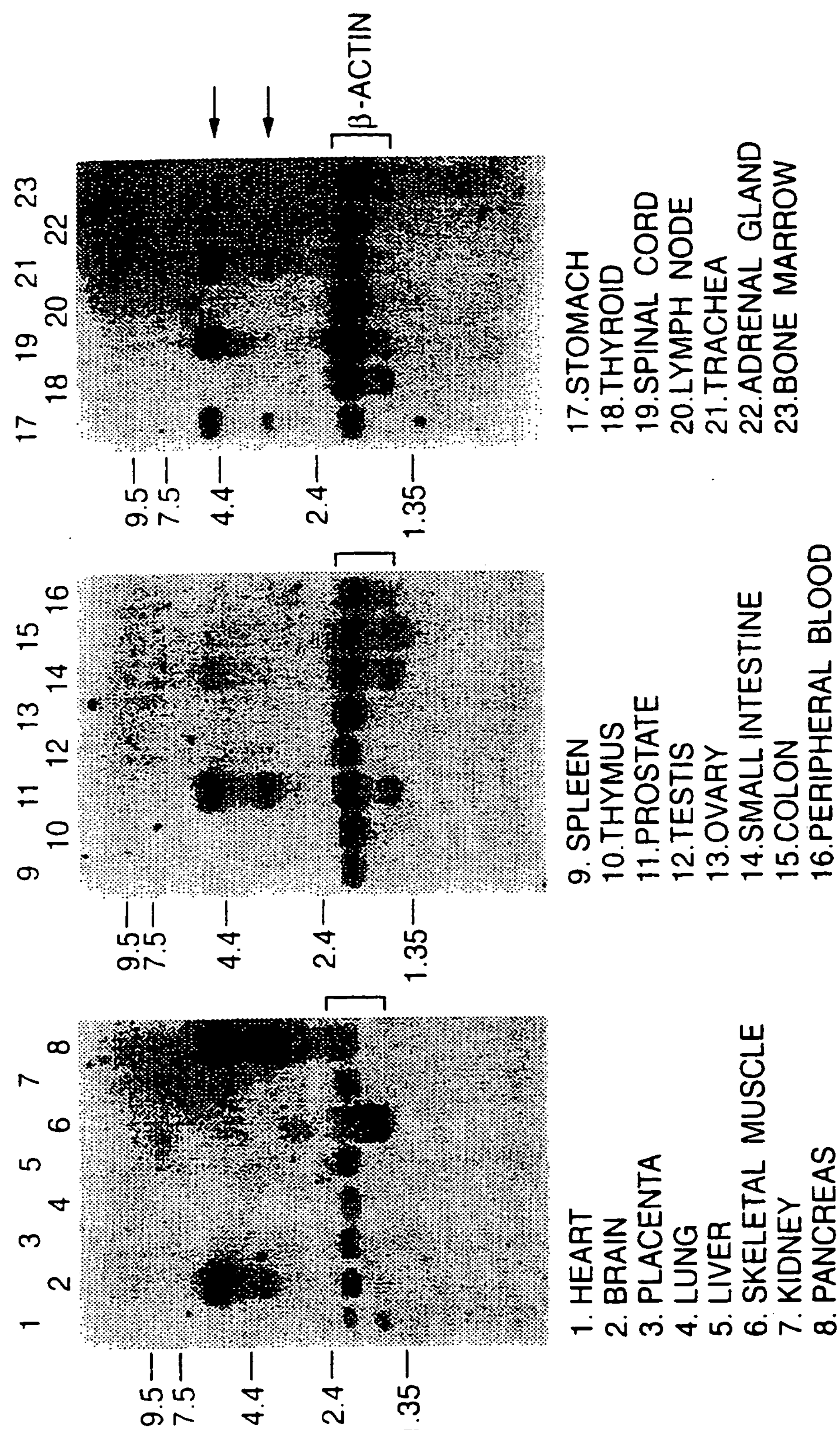


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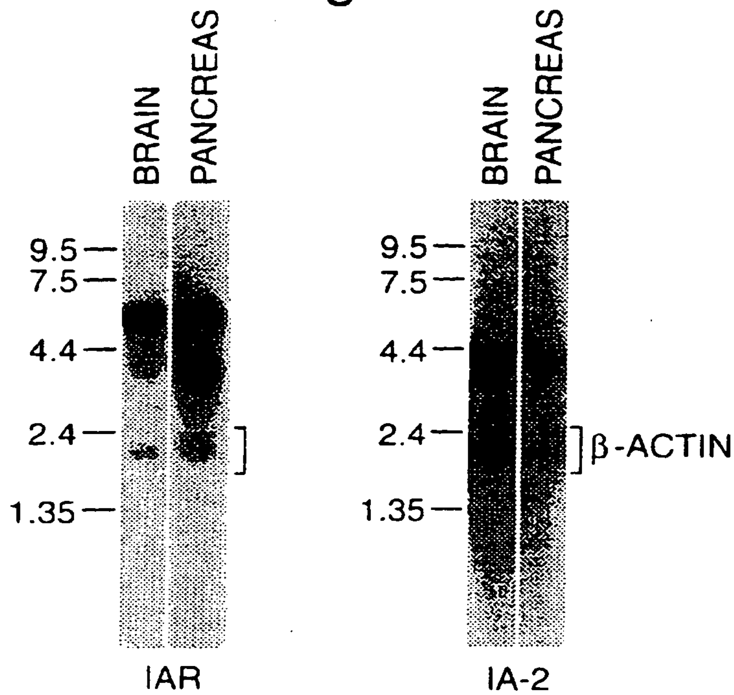
Fig.5.


BRAIN
PANCREAS
9.5
7.5
4.4
2.4
1.35
IAR
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PANCREAS
9.5
7.5
4.4
2.4
1.35
β-ACTIN
IA-2

Fig.7.

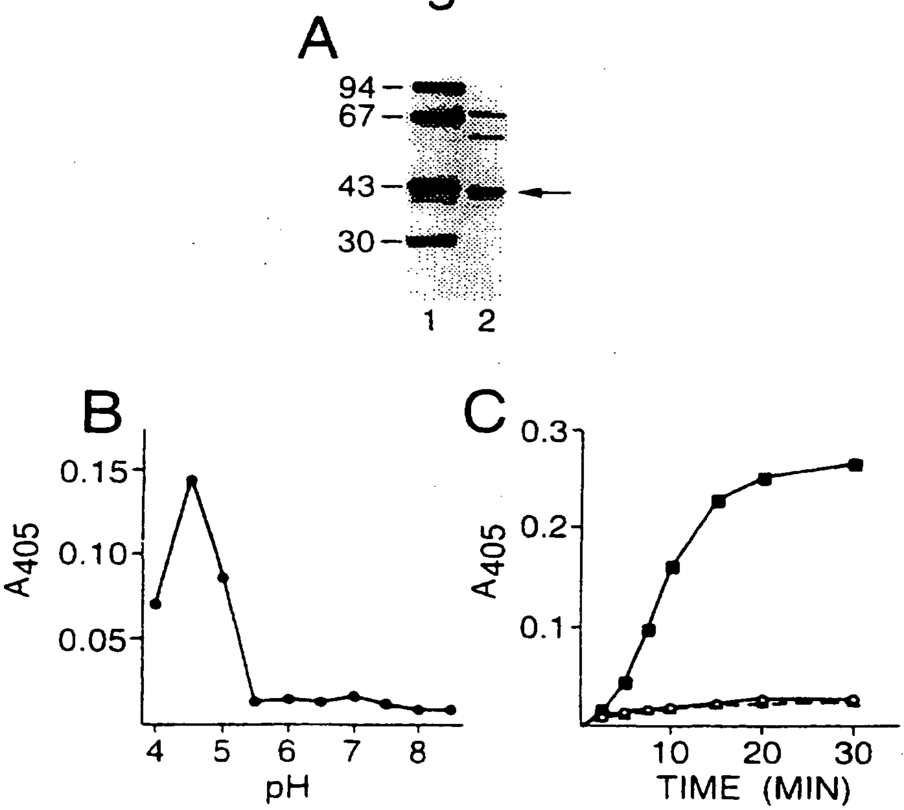
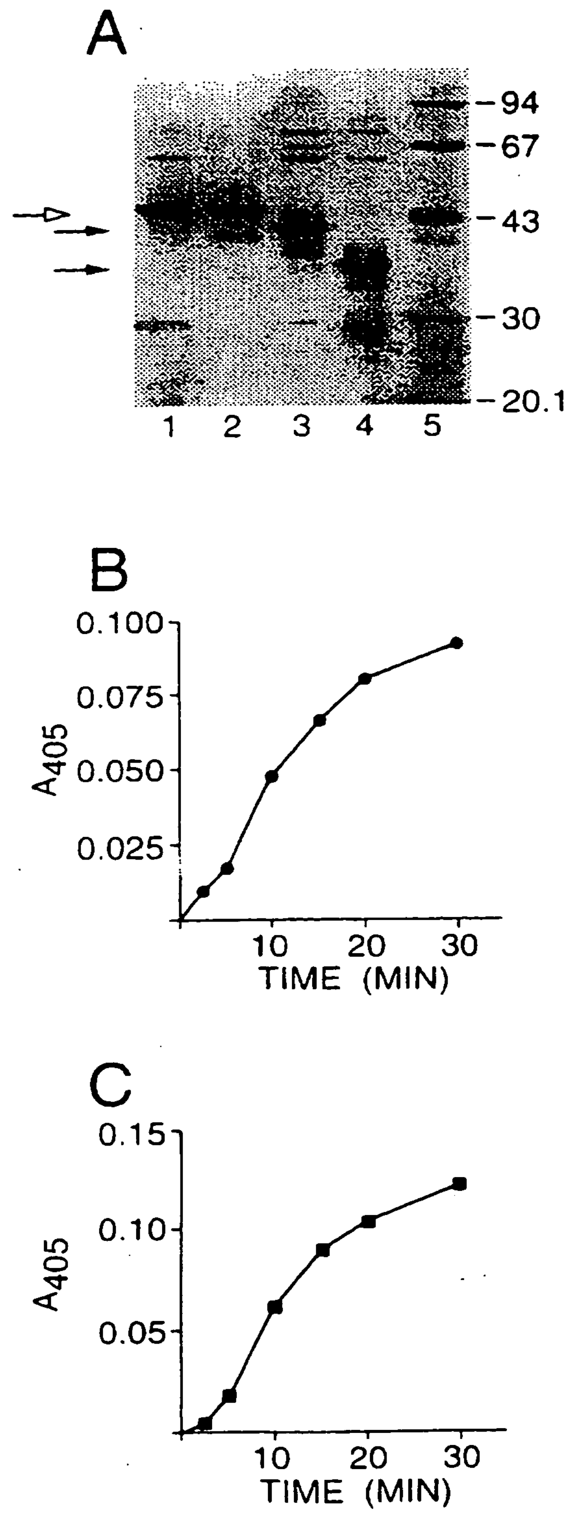

A
94
67
43
30
1
2
B
$A_{405}$
0.15
0.10
0.05
4
5
6
7
8
pH
C
$A_{405}$
0.3
0.2
0.1
10
20
30
TIME (MIN)

Fig.6.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67
43
30
20.1
1 2 3 4 5
B
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0.075
0.050
0.025
A405
10 20 30
TIME (MIN)
C
0.15
0.10
0.05
A405
10 20 30
TIME (MIN)

Fig.8.

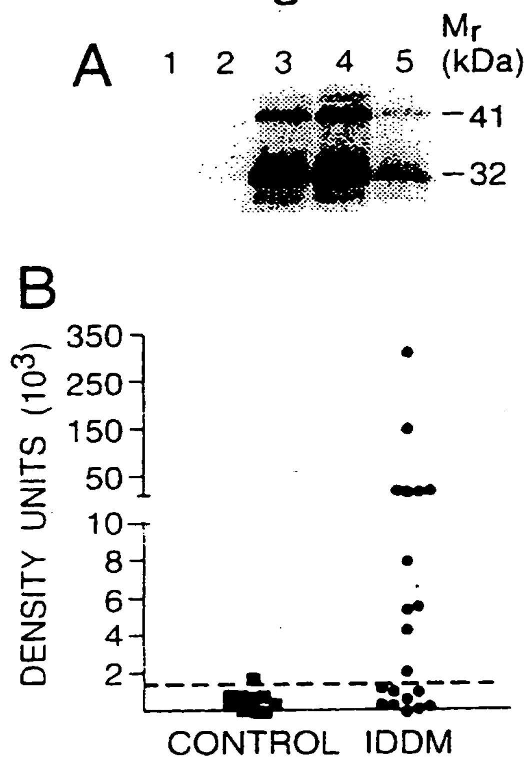

A
1 2 3 4 5
Mr (kDa)
41
32
B
DENSITY UNITS (10³)
350
250
150
50
10
8
6
4
2
CONTROL IDDM

Fig.9.

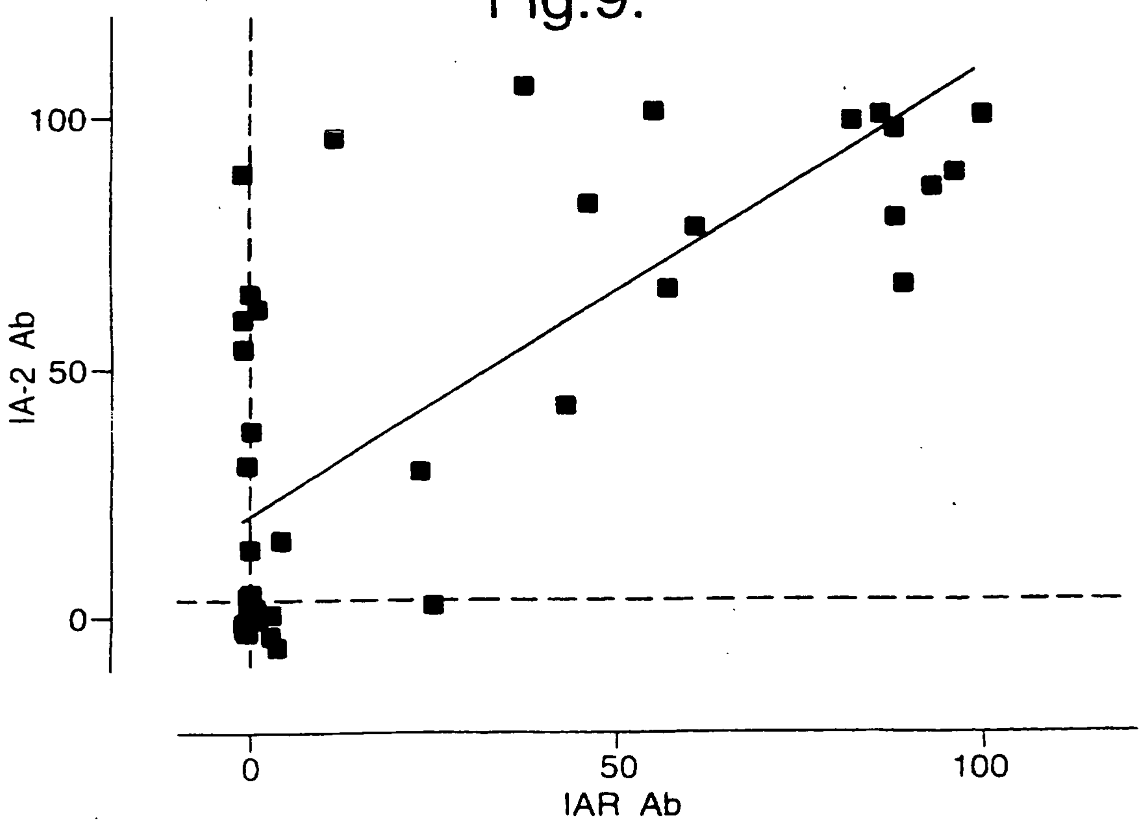
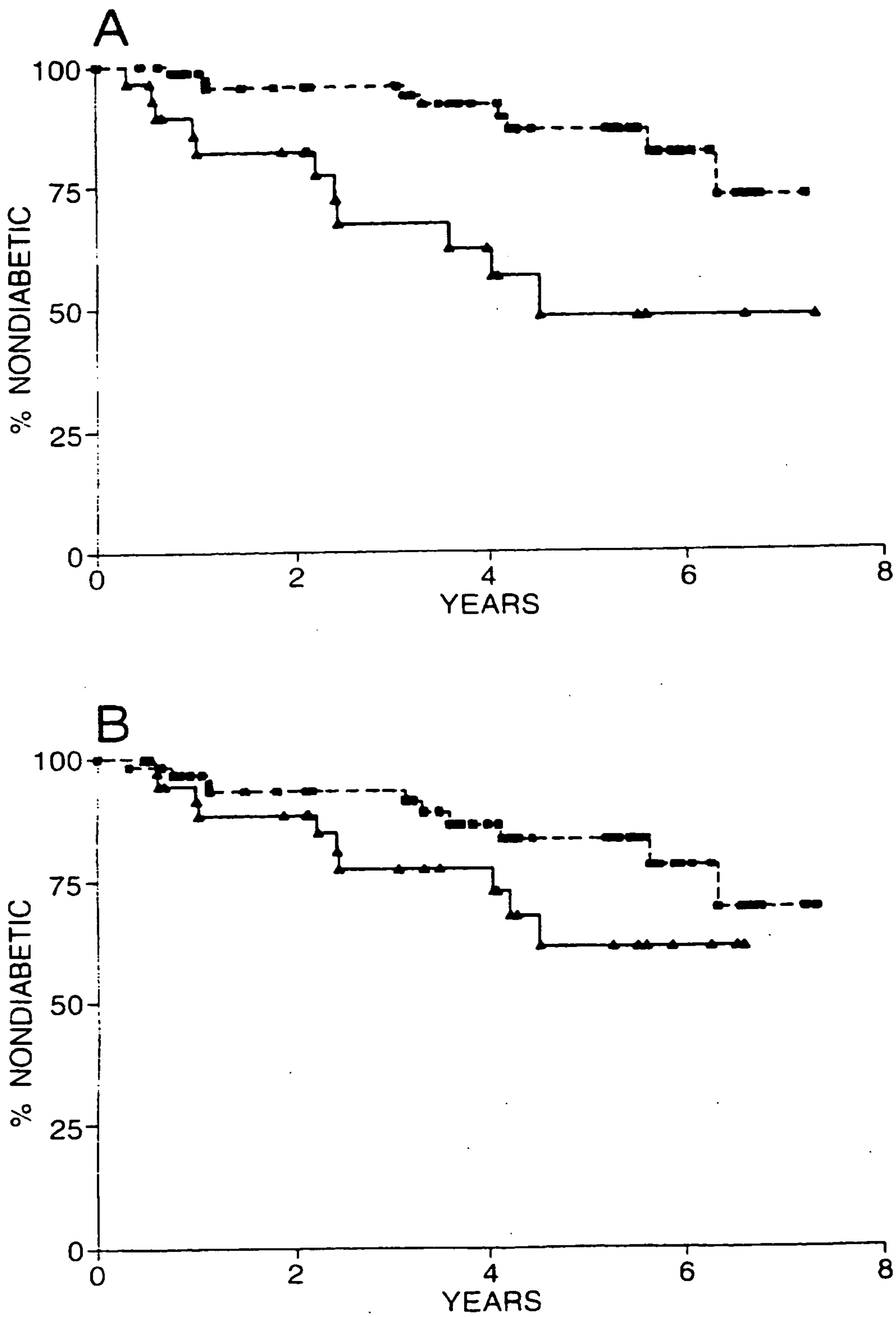

IA-2 Ab
100
50
0
0
50
100
IAR Ab

Fig.10.


A
100
75
50
25
0
% NONDIABETIC
0
2
4
6
8
YEARS
B
100
75
50
25
0
% NONDIABETIC
0
2
4
6
8
YEARS

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International Bureau

# INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

**(51) International Patent Classification** 6 :

**C12N 15/12, 15/55, 9/16, 5/10, C07K 14/47, G01N 33/564**

A3

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**(21) International Application Number:** PCT/CA96/00867

**(22) International Filing Date:** 20 December 1996 (20.12.96)

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| 9605710.4 | 19 March 1996 (19.03.96) | GB |
| 9620265.0 | 27 September 1996 (27.09.96) | GB |

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**(71)(72) Applicants and Inventors** *(for JP US only)*: PALLEN, Catherine, Jane [CA/SG]; 10 Kent Ridge Crescent, Singapore 119260 (SG). CUI, Lin [CN/SG]; 10 Kent Ridge Crescent, Singapore 119260 (SG). YU, Wei-Ping [CN/SG]; 10 Kent Ridge Crescent, Singapore 119260 (SG).

**(74) Agent:** MACRAE & CO.; P.O. Box 806, Station B, Ottawa, Ontario K1P 5T4 (CA).

**(81) Designated States:** AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**

*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

**(88) Date of publication of the international search report:** 14 August 1997 (14.08.97)

**(54) Title:** DIAGNOSTIC REAGENTS RELATING TO DIABETES

**(57) Abstract**

The present invention relates to a nucleic acid encoding a polypeptide that has the properties of an Insulin-dependent diabetes mellitus (IDDM)-associated autoantigen, which nucleic acid comprises: (a) the coding sequence of SEQ. ID. No. 1, 10 or 11, and/or the sequence complementary thereto; (b) a sequence which hybridises to a sequence defined in (a); (c) a sequence that is degenerate as a result of the genetic code to a nucleic acid sequence defined in (a) or (b); (d) a sequence having at least 80 % homology to a sequence defined in (a), (b) or (c). It also relates to the polypeptides encoded by such nucleic acids, and to diagnostic methods which employ such polypeptides.

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| GA | Gabon | MR | Mauritania | VN | Viet Nam |

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 96/00867

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/55 C12N9/16 C12N5/10 C07K14/47
G01N33/564

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | JOURNAL OF CLINICAL INVESTIGATION, vol. 96, September 1995, pages 1506-1511, XP000612574 PAYTON M.A. ET AL.: "Relationship of the 37.000 and 40.000 Mw tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512)." cited in the application see the whole document, especially abstract and p1511, lines 2-9 --- | 1-16 |

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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4

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 11 June 1997 | 01.07.97 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016 | Mandl, B |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | DNA AND CELL BIOLOGY, vol. 13, no. 5, May 1994, pages 505-514, XP000612611 LAN M S ET AL: "MOLECULAR CLONING AND IDENTIFICATION OF A RECEPTOR-TYPE PROTEIN TYROSINE PHOSPHATASE, IA-2, FROM HUMAN INSULINOMA" cited in the application see figure 4 --- | 1-16 |
| A | WO 91 17186 A (UNIV LEIDEN) 14 November 1991 see the whole document --- | 1-16 |
| A | NATURE GENETICS, vol. 4, no. 3, July 1993, pages 256-267, XP000674622 ADAMS M.D. ET AL.: "3400 new expressed sequence tags identify diversity of transcripts in human brain." cited in the application see page 261, right column, EST03250 --- | 1-7 |
| E | WO 97 07211 A (US HEALTH) 27 February 1997 see the whole document --- | 1-16 |
| P,X | JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 40, 4 October 1996, pages 24817-24823, XP002032721 CUI L. ET AL. : "Cloning and characterization of islet cell antigen-related protein-tyrosine phosphatase (PTP), a novel receptor-like PTP and autoantigen in insulin-dependent diabetes." see the whole document --- | 1-16 |
| P,X | BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 227, no. 2, 14 October 1996, pages 440-447, XP002032722 KAWASAKI E. ET AL.: "Molecular cloning and characterization of the human transmembrane protein tyrosine phosphatase homologue, phogrin, an autoantigen of type 1 diabetes." see the whole document --- | 1-16 |

-/--

4

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,O, X | 32ND ANNUAL MEETING OF THE EUROPEAN ASSOCIATIONN FOR THE STUDY OF DIABETES, DIABETOLOGIA, vol. 39, no. SUPPL.1, 1 - 5 September 1996, VIENNA, AUSTRIA, page a85 XP000612637 SEISSLIER J. ET AL.: "Autoantibodies to tyrosine phosphatases IA-2 and IA-2 beta in insulin-dependent diabetes mellitus." see abstract | 8-16 |

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4

:national application No.

PCT/CA 96/ 00867

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 12
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 12 is directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

..ormation on patent family members

Intern: ıl Application No
PCT/CA 96/00867

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9117186 A | 14-11-91 | NL 9001083 A | 02-12-91 |
| | | AT 144529 T | 15-11-96 |
| | | AU 652157 B | 18-08-94 |
| | | AU 7863291 A | 27-11-91 |
| | | CA 2082162 A | 05-11-91 |
| | | DE 69122864 D | 28-11-96 |
| | | DE 69122864 T | 05-06-97 |
| | | EP 0528886 A | 03-03-93 |
| | | JP 5508395 T | 25-11-93 |
| WO 9707211 A | 27-02-97 | AU 6896396 A | 12-03-97 |